Cloning, sequence determination and expression in E. coli of the gene for the human immunoglobulin epsilon chain expressed in a myeloma cell line

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CLONING, SEQUENCE DETERMINATION, AND EXPRESSION IN E.COLI OF THE GENE FOR THE HUMAN IMMUNOGLOBULIN ε CHAIN EXPRESSED IN A MYELOMA CELL LINE.

A Thesis submitted for the Degree of Doctor of Philosophy in the University of London.

BY

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July 1983.

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ABSTRACT

Messenger RNA has been isolated from cells of the human myeloma line 266BL which synthesizes IgE of the myeloma ND. A fraction enriched in mRNA for the ε heavy chain was copied into DNA and the DNA was cloned in Escherichia coli. A chemically synthesized oligonucleotide probe, based on the experimentally determined sequence of the specific message, was used to screen colonies. The largest ε chain cDNA cloned, 2.0 Kilobases, was characterized by restriction endonuclease mapping and DNA sequence analysis. It appears to encode the complete amino acid sequence of the mRNA. The missing part of the previously published amino acid sequence of the ND ε chain was determined from the DNA sequence.

Part of the cloned gene including the sequence coding for the second, third and fourth domains (CH_2, CH_3 and CH_4) of the immunoglobulin, has been coupled to the tryptophan control region of the expression plasmid, pWT211 and sub-cloned in E. coli. On induction of gene expression with 3-β-indole acrylic acid, the level of synthesis of the epsilon chain fragment reaches 18% of total protein synthesis, and yields of 55 mg/l of culture are obtained. The translation product has a molecular weight of 40,000 daltons, forming insoluble inclusion bodies within the bacterial cell.

Abbreviations

CPM	Counts per minute.
PBS	Phosphate buffered saline
	10mM Na_2PO_4, pH 7.4; 150mM NaCl.
Ac	Acetate
FCS	Fetal Calf Serum.
$NaDodSO_4$	Sodium Dodecyl Sulphate.
PMSF	Phenylmethylsulfonyl fluoride.
NEM	N-ethylmaleimide.
SSC	Standard Saline Citrate
	(0.15M NaCl, 0.015M Na Citrate)
EDTA	Ethylenediaminetetraacetic acid.
BSA	Bovine serum albumin.
Trp E	*E. coli* Tryptophan gene product E.
DMSO	Dimethylsulphoxide.
HEPES	N-2-hydroxyethylpiperazine
	N'-2-ethanesulfonic acid.
TMB	3, 3', 5, 5' Tetramethylbenzidine.
TCA	Trichloracetic acid.
Kb	Kilobases.
DH	Denhardts (0.2% Ficoll, 0.02% polyvinylpyrollidene and 0.02% BSA)

ACKNOWLEDGEMENTS

I would like to thank Dr. Hannah Gould, my supervisor, for the invaluable support and ideas during the course of the work; Dr. Mike Houghton for giving me so much of his time to help me while I was at G. D. Searle; Dr. Harold Molgaard for giving me encouragement and valuable criticism; Dr. Julia Warwick for valuable advice and help during cloning and RNA blots.

I wish to thank all of my friends and colleagues at the Biophysics Department and G. D. Searle also for their support.

Finally a special thank you for the typing to Mrs. Bridget Lister.

CHAPTER 1

INTRODUCTION:

1. Expression in bacteria of foreign genes

Bacterial synthesis of polypeptides in large amounts is a potential of great value. The synthesis of these polypeptides in large amounts is typically dependent on plasmid expression vectors. The expression of a cloned gene sequence in an expression vector requires that it first be transcribed and secondly that the transcript be translated. Thus, a plasmid designed for this purpose must contain a functional promoter and functional ribosome recognition site, known as the Shine and Dalgarno (SD) sequence (Shine and Dalgarno, 1975). The synthesis of eukaryotic proteins has been achieved in E. coli in two forms either by synthesis as a fusion with an E. coli protein, a so called chimera, or by itself by initiation at its own AUG initiator codon or one added by manipulation of the gene in vitro.

The expression by fusion of an E. coli gene, at the 5' end of the desired sequence, is employed as the E. coli gene is known to be expressed in the bacteria, and potential problems in the transcription or initiation of translation due to RNA primary or secondary structures are reduced. (Gold et al., 1981). This may not hold for 5' termini fusions with only a few bases, where secondary structures may form (Iserentant and Fiers, 1980). This use of chimeric prokaryotic/eukaryotic genes does not guarantee a high level of expression. The chimeric gene may have problems due to RNA secondary structure and stability, reducing the translation efficiency

(Iserentant and Fiers, 1980, Platt, 1981, Gottesman et al., 1982), proteolysis also may be a significant factor (Itakura et al., 1977) or codon usage may reduce the level of the expressed product in bacteria (Grosjen and Fiers, 1982). The problem of proteolysis may be overcome in certain cases by genetic manipulation to increase the length of the bacterial protein sequence (Itakura et al., 1977). The usefulness of these gene fusion systems has been extended by the construction of plasmids which encompass all three reading frames, using the following systems: The tryptophan promoter, Trp E gene (Tacon et al., 1980,) β-galactosidase (Charnay et al., 1978) and β lactamase (Talmadge and Gilbert, 1980). These systems have proved valuable, but for many purposes, the production of chimeric proteins is undesirable. The chimeric polypeptides if used in vivo could potentially elicit an immune response to the sequences of bacterial origin rendering the polypeptide inactive. If this response was extended to the eukaryotic polypeptide, it might lead to an auto-immune disease. The long term deleterious effects might be of concern in replacement therapies, e.g. with insulin treatment for diabetes. The addition of extra sequences to the polypeptides may result in inactivation, as was concluded for the attempted cloning of mouse dihydrofolate reductase (Chang et al., 1978).

The use of chimeric genes for expression of eukaryotic gene sequences has been the most common approach, with the tryptophan gene products (Williams et al., 1982), β lactamase (Villa-Komaroff et al., 1978, Seeburg et al., 1978) β galactosidase (Itakura et al., 1977) and MS2 polymerase (Küpper et al., 1981, Derynck et al., 1980).

The potential for removal of the bacterial protein segment has been explored. This has proved feasible and most applicable for short polypeptides lacking internal methionine residues, where cyanogen bromide cleavage at the methionine residue located at the junction of the chimeric polypeptide releases active protein, the methionine being lost (Goeddel et al., 1979a, Itakura et al., 1977, Wetzel et al., 1980). The use of this method of cleavage for large polypeptides is often impracticable as they are likely to contain internal methionines. The use of proteases has also been explored, e.g. trypsin cleavage of mouse β endorphin at an arginine residue, after the lysines were protected by chemical blocking prior to digestion (Shine et al., 1980). It is also possible that processing of the expressed protein could remove the bacterial NH_2 terminal sequences as was the fate of rat pre-proinsulin fused with up to 25 amino acids of β lactamase resulting in production of mature proinsulin protein and transport into the periplasmic space (Talmadge et al., 1980a, 1980b).

The use of expression vectors for production of proteins without bacterial sequences appears to be a better alternative as potentially only a single expression plasmid is needed. The disadvantages can be significant in that each cloned gene needs to be precisely tailored for insertion into the plasmid vector. If no restriction site is convenient, the construction of a synthetic gene segment is required, as with human growth hormone (Goeddel et al., 1979b) or a Bal 31 (double stranded exonuclease) digestion of unwanted sequence followed by screening using oligonucleotides to allow discrimination

at the variation of a single base (Wallace et al., 1979) allowing selection of the correct junction. The expression using these vectors is also apparently individually dependent on the distance between the SD sequence and AUG initiation codon, due to possible involvement of secondary structure in the RNA (Gheysen et al., 1982). With human growth hormone, expressed from a β lactamase promoter, the SD to AUG initiator codon was found to be the best at 11 base pairs (Goeddel et al., 1979b), but for expression of SV40 t-antigen a distance of 11 base pairs gave two fold lower expression than for a 9 base pairs distance. The second disadvantage is that expression of the protein from an AUG initiator codon located in front of the mature gene could result in production of the polypeptide with the N-terminal methionine residue still present, as not all bacterial proteins have this removed (Sarimo and Pine, 1969). In the long term, production of native proteins is the aim of many workers but initial studies are typically using the convenient systems provided by fusions to bacterial genes.

The nucleotide sequences involved in expression of eukaryotic genes in the prokaryotic system that have received most studies are those required at the 5' end of the gene. This region contains the site at which transcription starts under the influence of promoter sequences, and the site for ribosome binding in the mRNA prior to translation. The promoter sequences most used for expression of eukaryotic gene sequences are the leftward promoter (PL) of bacteriophage λ (Derom et al., 1982), the E. coli lactose operon (lac) promoter and the E. coli tryptophan operon (Trp) promoter (Roberts

et al., 1979; Goeddel et al., 1980), these being well characterised and strong promoters of transcription. Detailed studies of the PL, Lac and Trp promoters have been made by construction of convergent promoters. In the case of PL versus Trp, the expression from Trp was blocked but from PL only partially indicating the PL to be a stronger promoter (Ward and Murray et al., 1979). The study of convergent lac and trp promoters demonstrated that they were about equal in strength, but the lac was sensitive to the level of purine nucleotide, becoming relatively weaker at lower concentrations (Horowitz and Platt, 1982). With expression for human fibroblast interferon, the trp promoter proved to be about 2 fold better than the lac promoter (Goeddel et al., 1980). The expression of SV40 small t antigen by two groups indicated that the PL promoter activity was similar to the lac promoter (Derom et al., 1982; Roberts et al., 1979). The results with convergent promoters possibly provides the best analysis of transcriptional activity, as other factors may be involved in comparison of levels of expression obtained from these promoters. Clearly these promoters provide similar levels of transcription, with the possibility that the PL promoter is the best. The advantages of the PL promoter and the trp promoter are that they provide a good repression of transcription together with good levels of induction when required. Induction was effected with the PL promoter by a temperature sensitive repressor protein being inactivated at 42°C. With the trp promoter, tryptophan causes repression and ß indole acrylic acid (a tryptophan analogue) causes induction both via the repressor protein and the attenuator. These

properties of control for the PL and trp promoter (Remant et al., 1981; Hallewell and Emtage, 1980) are not found to the same degree for the lac promoter. This control would be of greater value for expression of toxic polypeptides, which after synthesis kill or disable the host.

The choice of the trp promoter in our case and production of a fusion polypeptide stemmed from the availability of the required expression plasmids (Tacon et al., 1980), with their demonstrated ability to synthesize large amounts of protein; up to 30% of total cell protein with 15% as a single gene product (Trp D) (Hallewell and Emtage, 1980). The use of these plasmid expression vectors and derivatives has been well characterised (Tacon, 1981).

The control of the tryptophan promoter has been the subject of extensive study. It is unusual among the biosynthetic amino acid operons of E. coli in having control by both repression and attenuation. The trp repressor protein responds to the level of trp in the bacteria and controls the initiation of transcription. The control by attenuation is by termination of transcription before the first functional gene. The role of attenuation in control of transcription of the trp operon is modulated via the tryptophan charging of tRNA. The termination or not of transcription at the attenuator site, is controlled by modulation of the termination signal in the RNA. *The termination signal consists of a GC rich

*Numbers and letters (n) in this discussion are those in figure 1-1.

hair pin structure (3,4) followed by a 3' terminal stretch of uridine residues at which termination typically occurs. The control of this termination structure in the mRNA is achieved by the presence of a further hair pin structure 5' of the termination hair pin. This hair pin 5' of the termination hair pin (1,2) contains within part of its 3' half (2), a sequence capable of forming a further hair pin structure with the 5' half (3) of the termination signal hair pin. In the absence of any other interactions, two hair pins form, causing termination (C). The prevention of termination is achieved by interaction of a ribosome with the RNA 5' to the first hair pin, (5' to the potential termination hair pin) at the initiation codon of a short peptide sequence of 14 amino acids. The ribosome then moves down the RNA translating the short peptide sequence until it reaches and covers the 5' half (1) of the first potential hair pin (5' to the potential termination hair pin). The ribosome at this point encounters two tryptophan codons, the ribosomes will pause if the trp-tRNA level is low this allows the 3' half of the first hair pin (2) to interact with the 5' half of the termination hair pin (3) before the termination hair pin can form. The result will be continued transcription (B). With high levels of trp-tRNA the ribosome will continue to disrupt the whole sequence involved in the first hair pin, preventing the 3' half (2) interacting with the sequence (3) for the termination hair pin resulting in termination of transcription (A) (see figure 1-1, for review Platt, 1981).

Work on ribosome binding sites has resulted in the accumulation

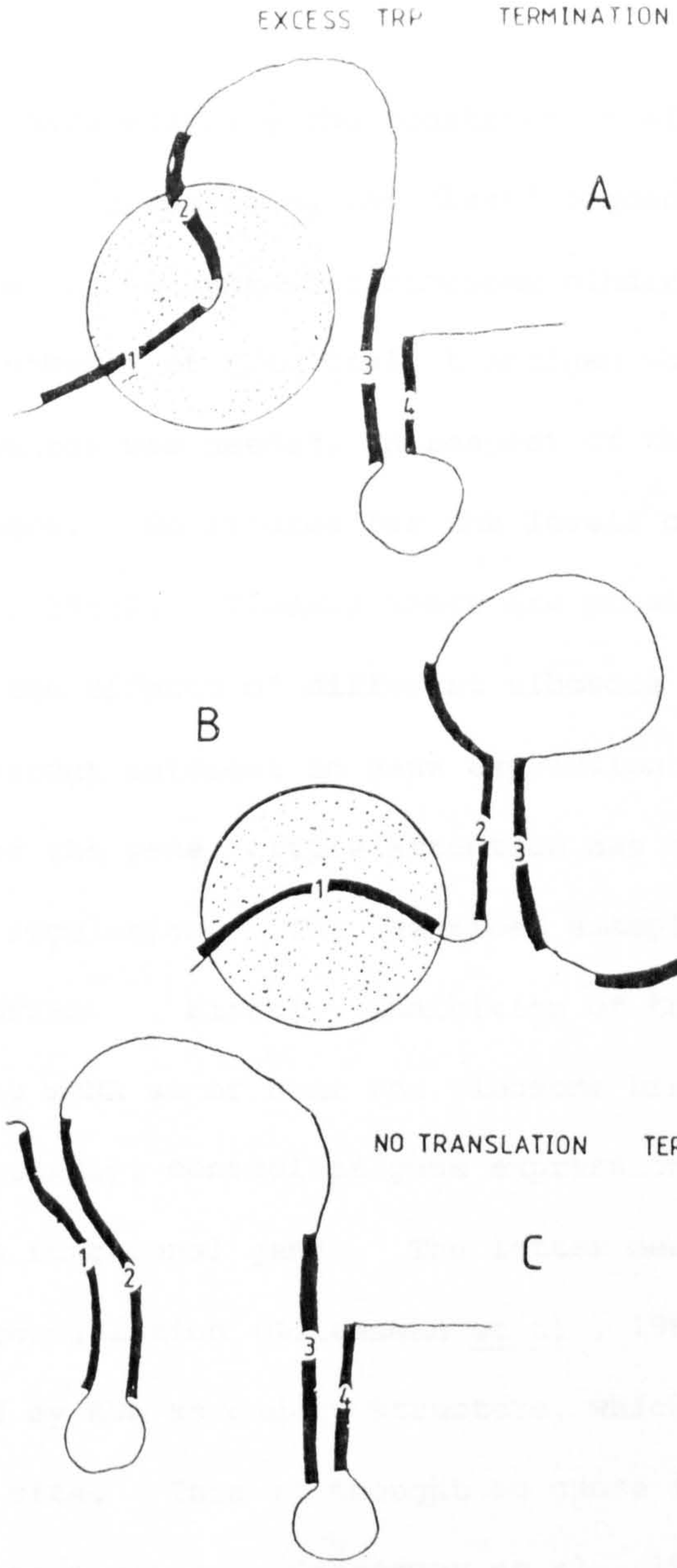


Figure 1.1 Model for Attenuation in E. coli Trp promoter/operator.

The ribosome is indicated as a dotted circle. The position of the ribosome on the RNA strand is indicated relative to a fixed position of the RNA polymerase, indicating the favoured structures formed by the RNA under the various conditions of amino acid availability. (See text for more detail.)

of sequence data enabling the construction of synthetic ribosome binding sites incorporating the 'best' sequences (Jay et al., 1980). The analysis of the synthetic ribosome binding sites for their ability to allow synthesis of SV40 small t antigen showed that modification to the sequences was needed, in respect of the SD to AUG initiator codon distance. No figures for the levels of synthesis were given (Jay et al., 1982). Clearly there are possibilities for further studies of the effects of different ribosome binding sites.

The current interest in gene expression has focused at the 5' sequences of the gene, little attention has been paid to post-transcriptional regulation. Two described examples suggest the latter may be important. Firstly, inhibition of translation by proteins that bind to mRNA at or near the ribosome binding site (Gold et al., 1981). Secondly, control of gene expression by sequences distal (3') to the functional gene. The latter means of control has been called retroregulation (Gottesman et al., 1982). It is thought to be mediated by RNA secondary structure, which creates a potential RNAase III site. This is thought to cause increased mRNA degradation by other nucleases (Gottesman et al., 1982). The results suggests that mRNA stability can be an important factor in control of expression. In brief, high level expression of eukaryotic gene sequences in bacteria requires: (1) a strong promoter of transcription; (2) an optimal ribosome binding site; (3) absence of secondary structure in the mRNA at the initiation AUG; (4) absence of transcription termination signals within the mRNA; (5) absence of retroregulatory sequences within the mRNA; (6) some degree of resistance to rapid proteolysis of the polypeptide.

11. Immunoglobulin E

Immunoglobulin E (Ig E) represents a class of proteins in serum of man and other mammals of minor abundance, typical human serum levels being 61-1000 µg/l (Bennich and Johansson, 1971). The importance of Ig E antibodies stems from their ability to mediate type I immediate hypersensitivity or reaginic hypersensitivity (Ishizaka and Ishizaka, 1975). This activity is due to their ability to bind reversibly with high affinity to specific membrane receptors on basophils and mast cells (Metzger et al., 1982). The main effect of the specific cell-bound antibody is to enable a triggering of a series of events which ultimately lead to the release of vasoactive amines with other pharmacologically active substances responsible for the clinical manifestation of hypersensitivity. The studies into the mechanisms of this process have demonstrated that firstly cross linkage of the receptor bound Ig E is the important effect in the allergic individual, this being mediated via the antigen or mimicked by the use of anti Ig E antibodies (Ishizaka et al., 1970), or mimicked by anti Ig E receptor antibodies (Ishizaka et al., 1978), causing histamine release. The detailed effects following stimulation via receptor aggregation have been examined. There is a rapid (15 second) transient peak in methylation of phospholipids, followed by an influx of Ca^{++} into mast cells and histamine release over several minutes. Inhibitors of methyl-transferase reactions prevented these responses (Ishizaka et al., 1980) these responses of the methyltransferase appear to involve a membrane bound protease (Ishizaka, 1982). The involvement of

arachidonic acid (AA) in the histamine release is found, it is also a precursor of the slow-reacting substance of anaphylaxis (SRS-A), one of the causative agents in the immediate hypersensitivity reactions (Murphy et al., 1979). Arachidonic acid is metabolised in two ways of interest, one way is through cyclo-oxygenase to e.g. prostaglandins and the other through the lipoxygenase pathway. Studies with inhibitors of both pathways have demonstrated a requirement for AA metabolites, for antigen, and for Ca^{++} ionophore induced histamine release from human basophils. This was also demonstrated by inhibition of phospholipase A_2 known to mobilize AA from phospholipid. Extension of these studies using selective inhibitors of the cyclo--oxygenase and lipoxygenase pathways showed that inhibitors of only the latter affect the antigen mediated histamine release, (Marone et al., 1981, Magro, 1982). This reveals the following outline of the process involved in the histamine release. Antigen first binds to the cell bound Ig E resulting in cross linking of the Ig E, and hence of the receptors. As a result methyltransferase is activated and phospholipids are methylated. Methylation of phospholipids apparently causes the opening of Ca^{++} channels, resulting in a Ca^{++} influx, followed by activation of phospholipase A_2 and metabolism of AA via lipoxygenase, resulting in histamine release (Figure 1-2).

To investigate the regions of the human Ig E involved in receptor binding, proteolytic fragments of purified Ig E have been prepared. The studies have been limited by the availability of sufficient Ig E and as a result most of the early studies have been

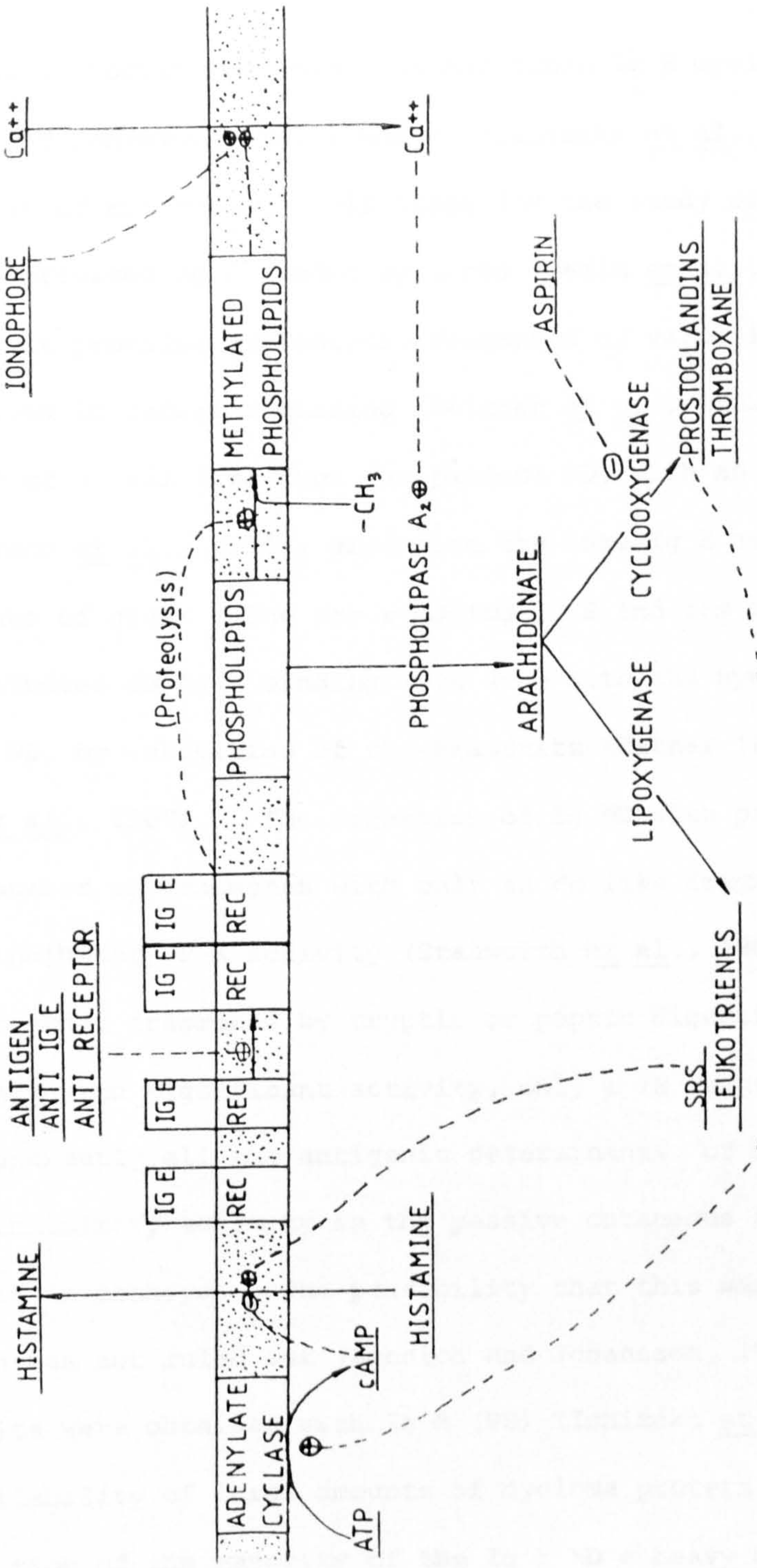


Figure 1.2 A scheme for the mechanism of basophil histamine release.

REC = Ig E receptors.

done in two laboratories who have obtained human Ig E myeloma proteins, ND, (Bennich and Johansson, 1971) and PS (Ishizaka et al., 1970). The development of rat myeloma cell lines for the study of immuno-globulins has provided Ig E producing lines (Bazin et al., 1974) but the Ig E has not provided proteolytic fragments of value in studying regions involved in receptor binding (Metzger et al., 1982). The establishment of a cell line from the patient ND, with an Ig E myeloma (Nilsson et al., 1970), producing the same Ig E as the patient is thus of great value for studying Ig E and its synthesis. The initial studies on Ig E binding were done with the myeloma protein Ig E ND, by inhibition of the Prausnitz-Küstner (P-K) test (Stanworth et al., 1967). The digestion of Ig ND with papain generated a number of fragments with only an Fc like fragment having significant inhibitory P-K activity (Stanworth et al., 1968). The generation of other fragments by tryptic or peptic digestion did not yield fragments with significant activity, only a 7S tryptic fragment containing apparently all the antigenic determinants, of intact Ig E and reduced inhibitory activity in the passive cutaneous anaphylaxis (PCA) reaction in monkeys. The possibility that this was due to contamination was not ruled out (Bennich and Johansson, 1971). Similar results were obtained with Ig E (PS) (Ishizaka et al., 1970).

The availability of large amounts of myeloma protein enabled the determination of the majority of the Ig E ND ε heavy chain amino acid sequence and many other parameters (Dorrington and Bennich, 1978). The determination of the molecular weight of the intact Ig E (ND) and (PS), and its ε heavy chain indicated that the heavy

chain of Ig E, like that of IgM was composed of 5 similarly folded protein regions (immunoglobulin domains). This was confirmed by sequencing (Figure 1-3), resulting in a schematic model of Ig E (Figure 1-4).

The availability of purified protein also allows the determination of physiochemical parameters associated with the loss of binding activity. The classical property of reagins, recognised long before the discovery of Ig E, was their sensitivity to elevated temperatures, (Coca and Grove, 1925). Circular dichroism (CD) studies have shown that structural changes do occur, and elaborated to include the study of three overlapping proteolytic fragments (pepsin F $(ab')_2$, pepsin Fc" and papain Fc) (Figure 1-4). The results showed that only the Fc fragment underwent an irreversible conformational change. With the Fc" containing part of the Fc region, (Figure 1-4) it was concluded that this effect was due mainly to the CH_3 and CH_4 regions of the Fc fragment (Dorrington and Bennich, 1978). This data agreed with the binding studies of these fragments (Stanworth et al., 1978; Ishizaka et al., 1970). Finer determination of the structure alterations has been obtained with a proteolytic fragment which has only lost the CH_4 domain, only small alterations in CD spectra are observed, possibly associated with the CH_3 domain. This indicates that the majority of changes occur in the CH_4 domain (Dorrington and Bennich, 1978).

The action of reducing agents on Ig E was also noted before its identification as a distinct class of immunoglobulin (Rockey and Kunkel, 1962). The disulphide bonds sensitive to reduction are

Figure 1.3 Amino-acid sequence of the epsilon chain of human Ig E (ND). Intra chain disulphide bonds are indicated by arrows (the additional disulphide in CH1 runs from CYS-139, or CYS-138 to CYS-225) and the inter chain disulphides are indicated by H (inter-heavy) and by L (inter-heavy-light). Oligosaccharide side chains are located at Asn-145, -173, -219, -265, -371 and -394. $F(ab')_2$ and Fc" fragments terminate at residues 339 and 338 respectively. The sequence of the peptide 330 to 334 contains one Asn,but its position has not been definitely determined. The sequence from 110 - 132 is based on homology considerations at that time (from Dorrington and Bennich 1978).

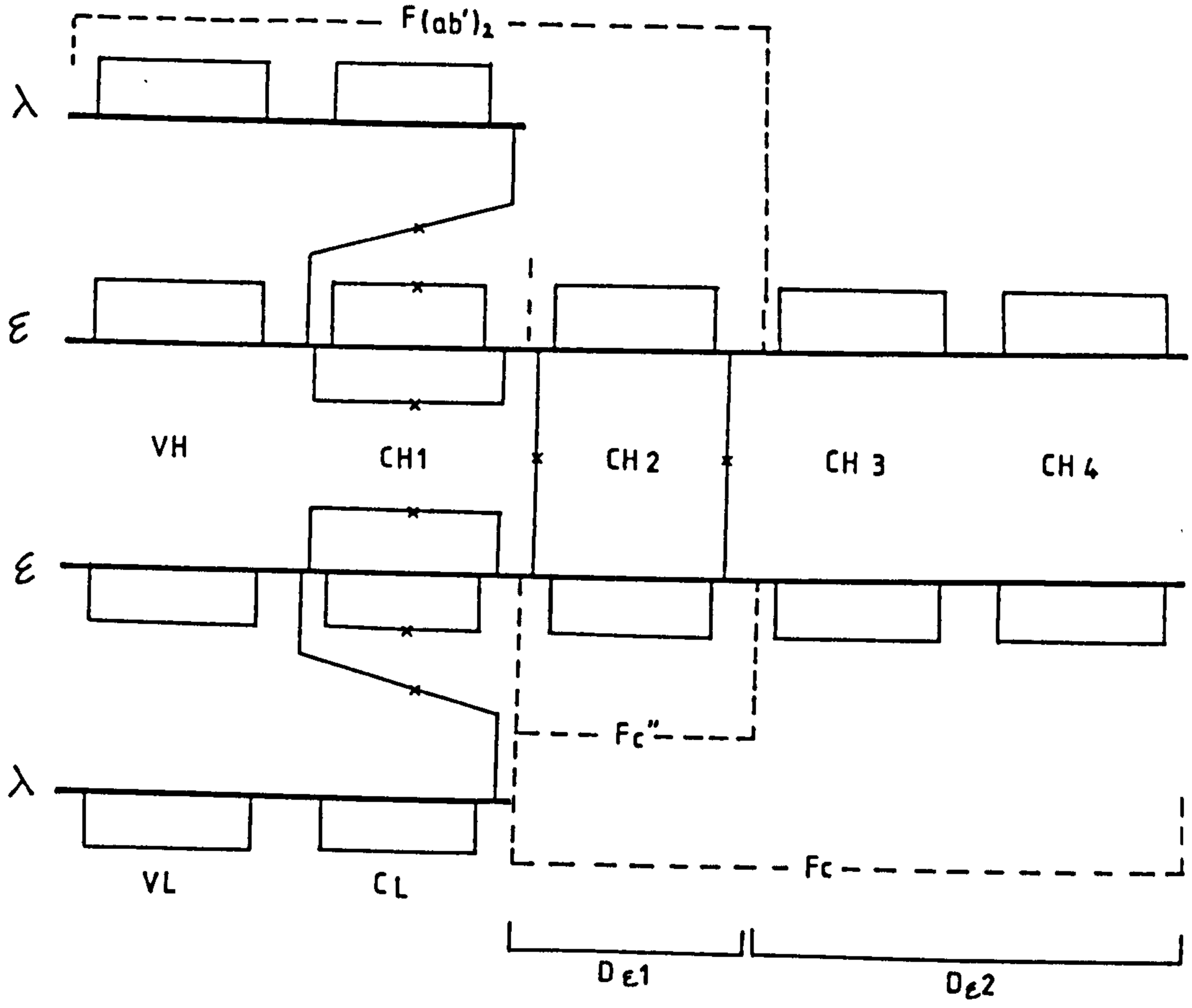


Figure 1.4 Schematic model of Ig E (λ) ND showing the molecular location of the various fragments used in the studies described. The thin lines represent disulphide bonds. Those marked X may be reduced with DTE in the absence of denaturing agents. Dε1 and Dε2 are two groups of antigenic determinants localized within the Fc region.

shown in figure 1-4, four being interchains bonds and four intrachain bonds. The two interchain bonds of interest between the heavy chains are at the NH_2 and COOH regions of the CH_2 domain, with the NH_2 located bond being the most resistant. The most extensive studies (Takatsu et al., 1975 and Dorrington and Bennich, 1978) indicate that of the two inter-epsilon disulphide bonds one is crucial to maintaining the receptor binding site. This bond probably is the one on the C-terminal side of the CH_2 domain, as it is more susceptible to reduction, the other remaining intact after loss of binding activity.

These studies have determined that an Fc fragment from Ig E is all that is required to explain the binding activity of Ig E to basophils and mast cells. These results, extension of earlier described characteristics of Ig E in response to heat ($56^{o}C$) and reducing agents (2-mercaptoethanol), suggested that the CH_4 region contains the binding site. Although the CH_4 regions do have an affinity for each other, tending to form a dimer (Dorrington and Bennich, 1978), the covalent linkage appears to be necessary for activity judging from the reduction data. As carbohydrate is absent from the CH_4 domain, these conclusions are supported by the finding of Kulczycki and Vallina (1981), that absence of carbohydrate had little effect on the binding efficiencies of rat Ig E. The latter authors also found that the ε heavy chain monomer was not active in binding.

These and other studies demonstrate a structure for binding activity of Ig E consisting of domains, CH_2 CH_3 and CH_4 linked as a

dimer, at least through the CH_2 domain COOH terminal disulphide. The evidence leads to a minimal polypeptide with receptor binding activity of: Part of the CH_2 domain with the COOH terminal inter-chain disulphide, CH_3 and CH_4 domains. Clearly in any bacterially synthesised products these structures would be desirable at the outset of any studies of Fc binding. Claims have been made that the binding of Ig E could be accounted for by the activity of a penta--peptide, apparently derived from the Ig E sequence (Hamburger, 1975). This penta-peptide with its incorrect Ig E sequence of Asp-Ser-Asp-Pro-Arg, instead of Asp-Ser-Asn-Pro-Arg, corresponding to residues 320-324 (Dorrington and Bennich, 1978), has been tested by other groups along with the correct Ig E sequence, and no evidence found to support this claim (Bennich et al., 1977).

111. Immunoglobulin genetics

Immunoglobulins contain 2 each of a light chain (κ or λ) and a heavy chain (from μ, δ, γ_3, γ_1, α_1, γ_2, γ_4, ε, α_2 (Flanagan and Rabbits, 1982a)) arranged essentially as in figure 1-3. Each chain has the COOH region constant in amino acid sequence, with the heavy chain type conferring the so called 'effector' functions on the complete immunoglobulin. The NH_2 termini of the light and heavy chains are characterised by a sequence of approximately 110 amino acids which has great variety, constituting the vast array of antigen binding sites generated by the immune system. Analysis of the immunoglobulin genes has demonstrated that the variable region is generated by somatic recombination of different gene elements (Hozumi and Tonegawa, 1976, Brack et al., 1978; for review see Gough, 1981a; Gottleib, 1980; Robertson, 1982; Shimizu et al., 1982). The arrangement of immunoglobulin genes in mouse germline DNA is shown (Figure 1-5), the human gene locus appears to be essentially similar but showing more complexity in the λ constant region gene locus and the duplication of the γ, ε and α genes (Hieter et al., 1981; Hieter et al., 1982; Max et al., 1982; Flanagan and Rabbitts, 1982a). The events occurring in the Ig heavy chain gene loci resulting in a functional gene element, serve to illustrate the general pattern of events necessary in the generation of functional light chain gene elements. The events involved in generation of a complete variable region, occur at the level of the germline DNA. Somatic recombination occurs between a large number, Ca ~300 variable (V) regions, an unknown number of

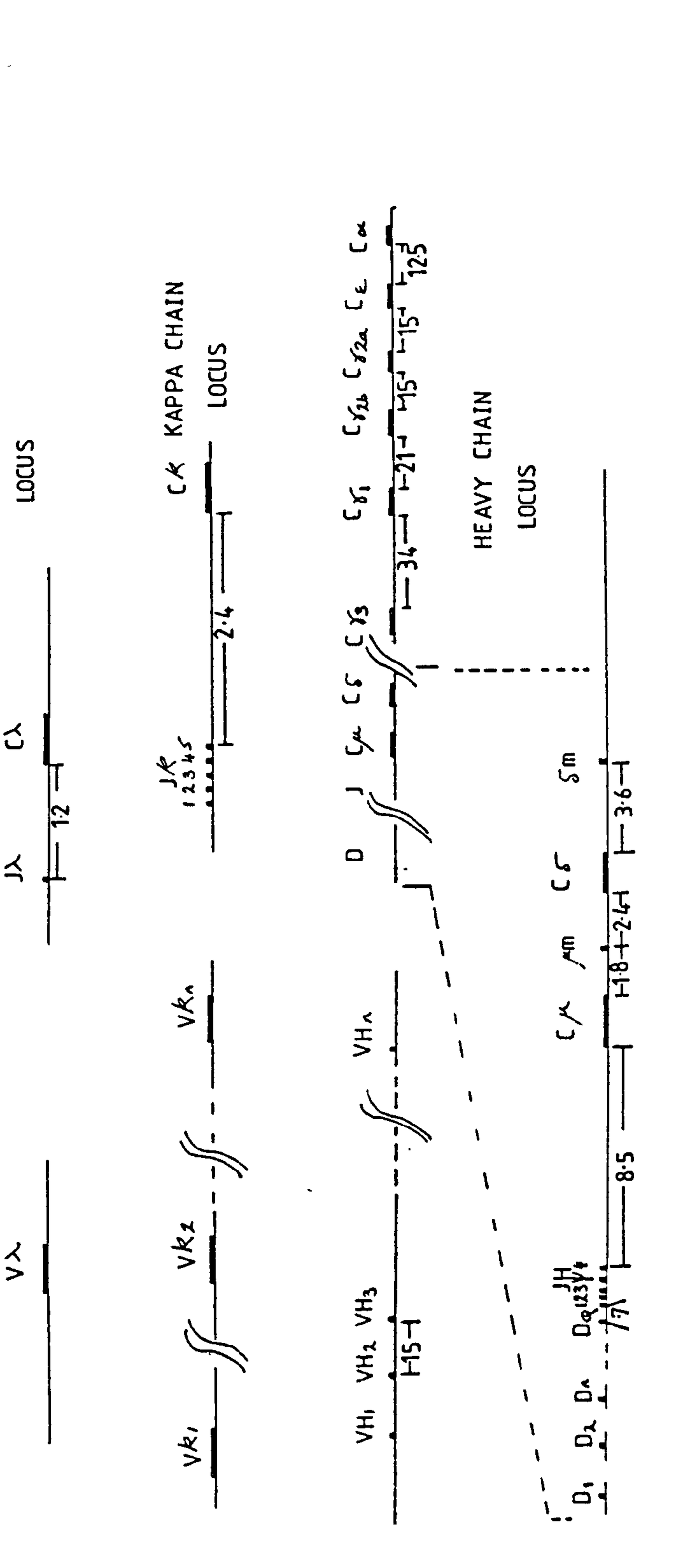


Figure 1.5 The arrangement of immunoglobulin genes in mouse germline DNA. The distances given are in Kilobases. JH ψ is a pseudo-J segment. DQ is the DQ52 gene segment (from Gough 1981a).

Diversity (D), regions, and 4 Joining (J) regions (Early et al., 1980; Sakano et al., 1981). These events also occur for light chains but in the absence of D regions (Bernard et al., 1978; Sakano et al., 1979). The accumulated sequence data has allowed the identification of sequences thought to be involved in these DNA re-arrangements. These signals consist of conserved nanomer and heptamer sequences paired, and separated by either 12 ± 1 or 23 ± 1 base pairs, the heptamer sequences being located closest to the gene segments. The consensus sequences for these signals is shown in figure 1-6 with the appropriate gene element. This arrangement of the sequences allows for a particular 'recognition' nanomer sequence, 5' GGTTTTTGT to always have the heptamer sequence all located 3', or for the other complementary nanomer 'recognition' sequence, the heptamer located 5'. This polarity would allow the same recognition systems to work on all the signals round the various gene elements constituting the complete Variable region (Sakano et al., 1981; Sakano et al., 1980). The spacer regions of these signals found in the immunoglobulin gene complex are arranged VK 12 ± 1 spacer, JK 23 ± 1 spacer; Vλ 23 ± 1 spacer, Jλ 12 ± 1 spacer; VH 23 ± 1 spacer, D flanked by 12 ± 1 spacer, JH 23 ± 1 spacer. This demonstrates that there is only recombination between signals with different spacers (Sakano et al., 1980; Sakano et al., 1981). These DNA joining events can produce variable region diversity by at least two means; (1) joining of germline gene segments and codon alteration at the site of joining (Weigert et al., 1978; Sakano et al., 1981; Sakano et al., 1979); (2) joining of any V germline gene segment to any J germline gene

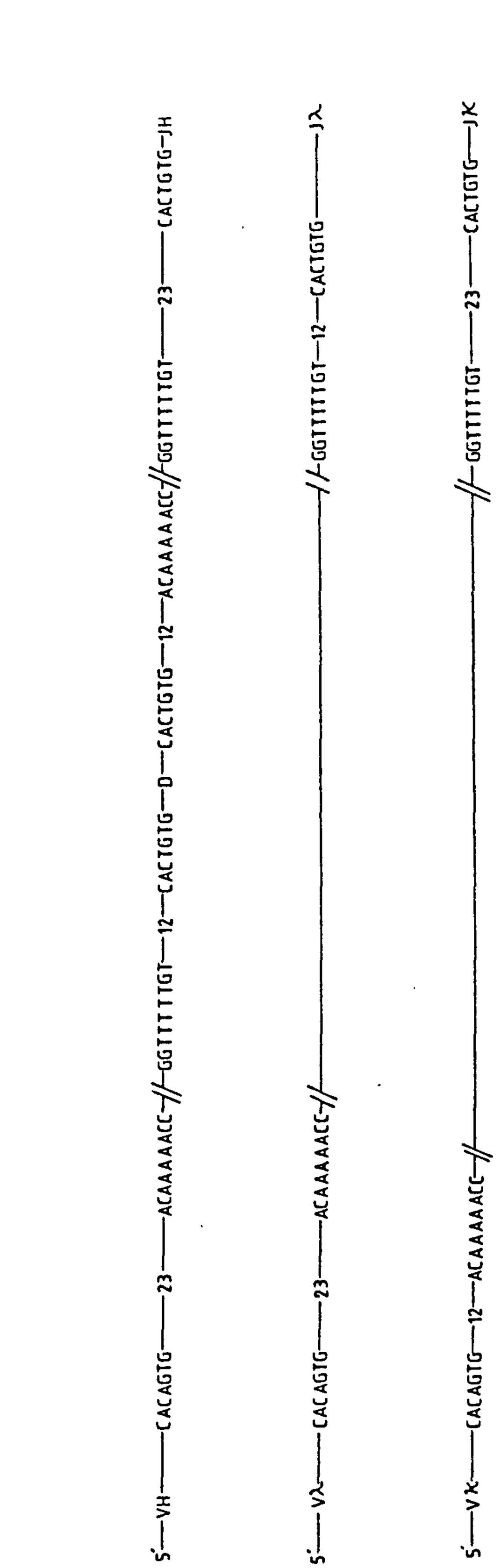


Figure 1.6 Schematic model of the arrangements of conserved sequences involved in recombination of the variable region gene elements of the light and heavy chains.

segments. Thus 200 VK and 4 JK gene segments could generate 800 Variable NH_2 terminal regions for expressed K light chains. The heavy chain gene segments involved in generation of the variable NH_2 termini, consisting of VH, D and JH clearly extend the possible diversity (Early et al., 1980). The generation of further diversity occurring at the level of combination between heavy and light chains (Gearhart et al., 1981). The possibility of generation of almost unlimited diversity is by a process of somatic mutation in the region of the recombined gene segment coding the variable region. This has been suggested as a possible fine tuning process for the antigen binding site (Gearhart et al., 1981). The cell at any time typically only expresses one heavy and light chain V region (allelic exclusion) although it may express this recombination with different heavy chain classes (Pernis et al., 1976). Of the light chains only K or λ is synthesised (isotypic exclusion), this may occur by a stochastic process of non-functional somatic recombinations (Gough 1981b). The selection of K or λ light chains, although they are separately complete genetic loci, appears to be a sequential event with K light chains being rearranged and utilized before λ light chains (Hieter et al., 1981). The rearrangement of heavy chain gene segments also occurs in a temporal sequence. After the creation of the rearranged functional variable region by recombination of the VH, D and JH gene elements, transcription of the μ gene element with the recombined Variable region is allowed, as inferred from Bentley et al., (1982) who showed for light chain VK region functional promoters are present. From the transcript the regions of intervening RNA are

spliced out to generate the processed RNA for translation into a μ heavy chain. The processing of the RNA transcript is thought to be responsible for the generation of 3 other heavy chain forms, μ with a membrane binding COOH terminal sequence, δ, and δ with a membrane binding COOH terminal sequence (Rabbitts et al., 1981). The other heavy chains are also thought to be capable of being expressed by this mechanism, e.g. ε which lies to the end of the heavy chain constant region (CH) gene cluster (Yaoita et al., 1982). This would require the production of a large mutli-CH transcription unit (~180 Kb for mouse) if it occured by this mechanism. Typically the expression of the other CH genes is thought to occur following a second recombination event mediated by so-called switch (S) sequences 5' of the μ heavy chain and other CH genes thus recombination has been demonstrated in most cases to result in the deletion of the intervening constant region gene segments and positioning of the other heavy chain genes for transcription and translation in the same manner as the μ constant region gene segment (Honjo and Kataoka, 1978; Sakano et al., 1980). This deletion of intervening sequences has enabled the mapping of the order of the CH genes, recently a complete gene locus has been isolated as overlapping genomic clones (Shimizu et al., 1982). The switch (S) sequences, unlike the signals involved in the recombination of the gene elements constituting the Variable region, are characterized by containing several common repetitive DNA sequences, e.g. (GAGCT)n (GAGAG)n and (GGCTG) (Dunnick et al., 1980; Kataoka et al., 1980). In the human chain constant region gene cluster hybridization occurs with

the mouse μ S sequences, indicating their conservation which gives strong evidence of their role in recombination of the CH locus. The nucleotide sequence data also obtained in the human CH gene locus shows the high level of homology to be found (Rabbitts et al., 1981).

Thus in brief for the differentiation of a lymphocyte to expression of Ig E (λ); there must have been a series of somatic recombinations involved in the formation of the Variable region, Vλ to Jλ joining (Sakano et al., 1979). This event possibly only occurring after initial deletion or re-arrangements in the K gene locus (Hieter et al., 1981). Also the somatic recombinations of the heavy chain variable region gene segments VH, D and JH (Early et al., 1980) must have occurred with the initial expression of Ig M (λ) (Pernis et al., 1976), followed by some mechanism for fixation of these variable region combinations most likely mediated via antigen interactions. This would then be followed by the switching of heavy chain class (Dunnick et al., 1980; Kataoka et al., 1980) and possible fine tuning of antigen binding by somatic mutation (Gearhart et al., 1981) to allow the production of Ig E (λ) and its fixation at this stage giving a stable immunoglobulin producing cell line in which no more alterations occur.

IV Molecular Cloning

The cloning of DNA by joining DNA molecules *in vitro* to vector DNA molecules followed by introductions into bacterial cells where they are multiplied is the bases of molecular cloning. The methods now available (Wu, 1979) allow the isolation of a desired sequence from a complex mixture of DNA molecules, e.g. as complex as the eukaryotic genome where the number of nucleotides for the haploid genome size is 2.8×10^9 base pairs (Lewin, 1974). Many applications have been found for this technology. The following are examples: 1. The DNA molecules can be multiplied easily and isolated for sequence determination; 2. The DNA sequences can be altered by various techniques (Zoller and Smith, 1982, Dalbachic - McFarland *et al.*, 1982); 3. Bacteria can be used to produce polypeptides encoded by the DNA on a large scale. (Itakura *et al.*, 1977). This of course excludes the direct use of genomic DNA containing introns such as found in most eukaryotic genes not excepting the genes for immunoglobulins (Hozami and Tonegawa, 1976). The goal of the present study was to clone and express in bacteria the DNA sequence corresponding to the constant region of human Ig ε heavy chain. This required the isolation of DNA copies of human Ig ε mRNA, and its use as a template for the synthesis of the desired coding sequence. We were fortunate in that an Ig E producing myeloma cell line had been established from a myeloma patient (ND). The cell line 266BL was originally dependent on a feeder layer of fibroblasts (Nilsson *et al.*, 1970). The recent adaptation to suspension culture (Dr. M. Steel, personal communication) greatly facilitated

production of the cells in sufficient numbers for preparation of the required mRNA. Ig E is only 0.1% of the Ig G level in serum; thus the use of normal human lymphocytes did not present a practical alternative.

The copying of mRNA into complementary DNA (cDNA) particularly using AMV reverse transcriptase has been the subject of much investigation because it is a key step in cloning and initially failed to give complete copies of cDNA (Ross et al., 1972). The results of these studies (Efstratiadis et al., 1975; Monahan et al., 1976; Buell et al., 1978) have been the basis of the success in cloning many genes (Gough et al., 1980; Suggs et al., 1981, for a list of cloned genes see Grantham et al., 1981). Much of the initial variability was possibly due to differing levels of ribo-nuclease in the different systems (Buell et al., 1978). The first method used in the present study involved a combination of procedures (described by Monahan et al.,(1976) and Buell et al.,(1978). This method was then discarded in favour of the more efficient one of Gough et al., (1980).

We used a plasmid vector for the cloning of our cDNA. This offered the greatest versatility for our purpose and its purification from the chromosomal DNA, RNA and proteins of E. coli is straight-forward. The plasmid vector chosen for this study was a derivative of pBR322 (Bolivar et al., 1977), viz pAT153. The parent plasmid pBR322 is a widely used and characterised cloning vector used by many workers (Gough et al., 1980; Nagata et al., 1980; Suggs et al., 1981). The plasmid pAT153 constructed by Twigg and Sherratt (1980)

has a deletion from nucleotide 1647 to 2352 (Sutcliffe, 1979) of two Hae II fragments (705 base pairs). The plasmid has an advantage over pBR322 with a copy number 2.7 times higher, and is non-mobilizable due to loss of the nic site. This makes it a better and safer cloning vector (GMAG Note 9) than pBR322.

DNA fragments can be inserted in three ways, two of which are essentially the same. Having opened the closed circular plasmid vector with an appropriate restriction enzyme, the simplest method is blunt-end ligation. This makes use of the ability of T4 DNA ligase to join DNA molecules with duplex, base-paired termini. The disadvantages of this technique are that unless the joined DNA ends reconstitute a restriction site, as would be the case for joining of two halves of a restriction endo-nuclease site, the whole DNA cannot generally be excised without additional plasmid sequences. The efficiency at low DNA concentrations is moreover low. This reaction is useful for addition of synthetic DNA containing restriction sites (linkers) to DNA molecules, and for manipulation of previously cloned sequences. Here the concentration of the reactants can be raised to make the ligation efficient.

The use of homopolymer tails is a very common way of obtaining insertion into the plasmid vector (Gough et al., 1980). The enzyme terminal deoxy nucleotidyl-transferase (terminal transferase) is used to add nucleotides to the 3' end of the vector, and complementary extensions to the 3' ends of the DNA to be cloned. The two species are mixed, heated and annealed before use in transformation of E. coli to allow hybridization of these extensions. The use of dA

and dT tailing allowing removal of the insert by partial denaturing with SI nuclease digestion has largely been replaced by the use of dG and dC tailing. This is due to the availability of restriction enzymes for removal of G, C tailed inserts. The Pst I site of the plasmid vector is tailed with dG, and the DNA to be cloned is tailed with dC, resulting in reconstitution of the Pst I site to allow excision of the cloned DNA.

Another method for insertion of cloned DNA into a vector is similar to the method using complementary homopolymer tails and also blunt end ligation. The method relies on the restriction enzymes that produce staggered cuts in DNA. These produce short specific single stranded sequences called cohesive ends which can be specifically ligated with the use of T4 DNA ligase. The ligation process is usually efficient. The method has the problem that the vector can ligate to its own ends resulting in exclusion of the DNA to be cloned. This self ligation problem is diminished by the treatment of the vector, after restriction endonuclease digestion, with bacterial or calf alkaline phosphatase to remove the 5' PO_4's to prevent self ligation of the vector preventing self ligations in blunt end ligations (Fiddes and Goodman, 1979).

In order to use ligation on DNA generated from mRNA which would be blunt ended, synthetic DNA linkers (blunt ended duplexed DNA molecules 8-14 base pairs containing restriction sites) are usually added by blunt end ligation at high concentration. Excess linker and half linker are removed by digestion with the appropriate restriction endonuclease (Fiddes and Goodman, 1979). The method

chosen for the cloning of Ig E mRNA made use of Hind III linkers essentially as described by Fiddes and Goodman (1979). Hind III linkers were chosen because from the available amino acid sequence data (Dorrington and Bennich, 1978), it could be predicted from a computer program (Dr. J. F. Pardon) that no Hind III restriction sites occur within the constant region. The cloning of the Ig E heavy chain with Hind III linkers would also allow the insertion, subsequent to characterization of the gene into the available expression plasmids (Tacon, et al., 1980). These expression plasmids contain a unique Hind III site to allow expression in all possible reading frames.

The E. coli chosen for initial cloning attempts was MRC8 (dap 103, hsd R, Met B1, glm 533, upp 1, dap 101, sup E, thy A 103, deo, rec A1; GMAG Note 9 - supplement 1) as this host in conjunction with the nic$^-$ plasmids of pAT153 allow the use of a lower category for recombinant work. MRC8 is a E. coli K12 mutant which requires in addition to L Broth supplementation with thymine, N-acetyl glucosamine and diaminopimelic acid. Transformation rates with this bacteria, although slow growing (a 40 minute doubling time), were typically 10^6 per µg of supercoiled pBR322. WT217 and HB101 were used for subsequent transformations for expression studies. The E. coli were rendered competent by Ca^{++} shock (Cohen et al., 1972).

Identification/Screening is commonly the limiting factor in the cloning of genes. For many genes which have been cloned long tedious procedures were used, involving clones or pools of clones to hybridize mRNA, which is then translated. The translation products

are identified by the use of antibodies if possible or by bioassay as in the case of human leukocyte interferon (Nagata et al., 1980). Alternative methods of screening are thus of great value. The use of complementary synthetic oligodeoxynucleotides has been a development which has contributed significantly to simplification of detection methods, many colonies (1×10^5) being readily screened. The use of oligodeoxynucleotides is especially valuable where the available antisera will not react with the translation product of mRNA, as in the case of gastrin mRNA (Noyes et al., 1979). The value of a highly specific cDNA probe generated by enzymatic extension of an oligodeoxynucleotide on mRNA was recognised in the screening for a full length clone for insulin mRNA (Chan et al., 1979). This use of oligonucleotides has been extended to allow the use of synthetic oligonucleotides as hybridization probes without enzymatic extension (Suggs et al., 1981). The use of oligonucleotide probes is only limited by the availability of protein sequence data for obtaining the possible nucleotide sequence, which may become more readily available with the use of monoclonal antibodies (for purification) and improved protein sequencing methods. Protein sequence data was fortunately available for the large part of the human Ig ε. This allowed the design of oligonucleotides for primer directed cDNA synthesis. The sequence data obtained was used to construct an oligonucleotide probe successfully employed to screen cDNA clones. Characterization of one clone, and sub-cloning followed by expression of a fragment corresponding to CH_2, CH_3 and CH_4 of the Ig ε chain in E. coli are described in this thesis.

Chapter 2: Materials and Methods

(1) Human Cell Culture

Ampoules of the cell line 266BL, stored in liquid nitrogen were thawed in a water bath at 37°C. The cells were resuspended in culture medium, RPMI 1640, pH 7.4 modified as described by Birch et al. (1979), and spun down to remove the dimethylsulphoxide (DMSO) used for storage of the cells. The cells were then resuspended in culture medium as above supplemented with 10% v/v fetal calf serum (Gibco), at a cell density of 10^5 cells/ml. The cultures were grown in disposable Roux bottles (750 mls, Gibco) at 37°C in an atmosphere of 5% CO_2. The cultures were maintained by observation of the cells and monitoring the pH of the medium.

Preparation of RNA

(2) Isolation of Microsomes. The frozen cell pellets were broken up and pulverized with a pestle and mortar under liquid nitrogen. The resulting powder was allowed to warm up to 0°C and suspended at 10 ml/g of cells in homogenization buffer (cf Marcu et al. (1978) containing 0.25 m sucrose in TKM (10 mM Tris-HCl, pH 7.5, 25 mM KCl, and 10 mM $MgCl_2$), plus 7 mM 2-mercaptoethanol, 0.2 mM cycloheximide, 0.25 mg/ml spermidine tetrachloride, and 1 mg/ml Heparin).

The cells were hand homogenized in a tight-fitting glass homogenizer (40 strokes). The nuclei and cell debris were removed from the homogenates by centrifugation 2 times for 5 minutes at 1,100 g (3000 rpm in a Sorvall SS34 rotor). The microsomes were recovered from thepost-nuclear supernatant by centrifugation through a 5 ml, 0.75 M sucrose cushion in TKM for 40 min at 50,000g (20,000 rpm, SS34 rotor).

(3) Isolation of RNA from Microsomes

Microsome pellets were suspended in the RNA isolation buffer (0.1 M NaCl, 0.01 M Tris-HCl pH 7.5, 0.001 M EDTA, 0.2% (w/v) sodium dodecyl sulphate (Na-Dod SO_4), and 500 µg/ml proteinase K (Boehringer)) and allowed to stand at room temperature for 20 minutes before shaking the suspension with a 1:1 phenol chloroform mixture, equilibrated with 0.1 M NaCl, 0.01 M CH_3COO Na, pH 6.0 and 0.001 M EDTA, as described by Perry et al. (1972). The final aqueous phase was precpitated with 2.5 volumes of ethanol, washed 2 times with 2 ml of 3M CH_3COO Na, pH 6.0, and reprecipitated with ethanol, as described by Palmiter (1974), before dissolving in sterile water.

(4) Isolation of RNA from Cells

(A) RNA was isolated directly from cells using guanidinium thiocyanate following a procedure, recommended by Dr. P. Butterworth (unpublished experiments), modified from that of Chirgwin et al. (1979). A 4 M guanidinium thiocyanate (Fluka) solution, containing 0.1 M 2-mercaptoethanol and 0.1 M potassium acetate, was adjusted to pH 5.0 with glacial acetic acid and cleared by 5 minutes of centrifugation at 50,000 g (20,000 rpm, SS34 rotor). Cells pulverized as described above for the microsomal preparation, were added to the guanidinium thiocyanate solution (10 mls/gm of cell pellet) and homogenized for 45 seconds in a Virtis homogenizer at setting 40. The homogenate was layered over a 1 mL cushion of 5.7 M cesium chloride containing 0.1 M EDTA, pH 7.5, and centrifuged at 185,000 g (33,000 rpm, SW41 rotor) at 15°C for 18 hrs. The supernatant and cushion were removed and the sides of the tubes were

washed and drained, the RNA pellets were suspended in 0.5 M NaCl, lO mM Tris-HCl, pH 7.5, 2 mM EDTA, and 0.2% w/v Na Dod SO_4.

(B) RNA was also isolated directly from fresh cells by using a high pH lysis buffer, followed by phenol/chloroform extraction (phenol/pH 9 method (Houghton et al., 1980a)). The suspension of cultured cells was spun down in an MSE mistral 6L at 3,000 rpm for 15 minutes. The lysis buffer (0.2 M Tris-HCl pH 9, 50 mM NaCl, 0.5% w/v Na Dod SO_4, lO mM EDTA) with 1 mg heparin/ml added fresh before use, was added directly to the cell pellets after removal of the supernatant. The cells lysed immediately and the viscous lysate was mixed with a glass rod to disperse the remaining cells.

This mixture was then homogenized in a tight-fitting glass-glass homogenizer (40 strokes). This mixture was then extracted with 2 volumes of phenol/chloroform (1/1, v/v) saturated with the above lysis buffer without added heparin. The phenol/chloroform was removed and interface re-extracted. This was continued until the interface after centrifugation at 15,000 g (Sorvall GSA 10,000 rpm) was minimal. The lysate was finally extracted 3 times by 2 volumes of chloroform. The nucleic acid from this lysate was precipitated by 3 volumes of ethanol at -20°C overnight. The nucleic acid was collected by centrifugation 10,000 g (Sorvall HB4, 10,000 rpm), and washed 2 times with 95% v/v ethanol. The nucleic acid pellet was dried under reduced pressure and the redissolved in 20 mM HEPES pH 7.5. The RNA was precipitated by addition of NaCl to make the solution 4M. This was left overnight at -15°C to allow complete precipitation of the RNA. The RNA was collected by centrifugation

as above, and washed with 3 M CH_3COONa pH 6.0 in order to remove residual DNA. The washed pellet was rewashed twice with 95% v/v ethanol to remove the CH_3COONa, then dried under reduced pressure and resuspended in 5 mM HEPES pH 7.5.

(5) Isolation of Poly A+ RNA using Oligo (dT)$_{12-14}$-Cellulose

Poly A+ RNA was isolated by oligo (dT) cellulose (using oligo (dT)-cellulose type T3 (Collaborative Research, Waltham, MA) or T-1 (Boehringer)) or type 7 (P-L biochemicals inc) essentially as described by Faust et al. (1979). Prior to application of the RNA the oligo (dT)-cellulose was first washed with 0.5 N KOH and then sterile water to remove any potentially contaminating RNA. The oligo (dT) cellulose is also washed with 0.1% v/v diethylpyrocarbonate in order to inactivate any ribonucleases. The oligo (dT) cellulose was washed and equilibrated with 0.5 NETS (0.5 M NaCl, 1 mM EDTA, 0.2% w/v Na Dod SO_4), 10 mM Tris-HCl pH 7.5. The RNA sample in 5 mM EDTA is heated to 65°C for 3-5 minutes, cooled in ice, made up to 0.5 NETS and loaded on to the oligo (dT) cellulose.

The unbound RNA is washed off with 0.5 NETS followed by 0.2 NET (0.2 NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA) until the transmission at 260 nm reaches 100% (Uvicord). The poly A+ RNA is eluted with sterile water. In earlier preparations the first pass poly A+ RNA was rerun following the above procedure leaving out the KOH and dethylpyrocarbonate washes.

Poly A+ RNA was also isolated by the use of a batch method using oligo (dT) cellulose (Searle Research Products); and Type 7 (PL Biochemicals Inc). The procedure is as described above except no

Na Dod SO_4 was used, KCl replaced NaCl, 5 mM HEPES pH 7.0 replaced 10 mM Tris-HCl pH 7.5. The intermediate salt wash was with 0.1 M KCl. poly A+ RNA eluted with 5 mM HEPES pH 7.0. The poly A+ RNA was precipitated after making the eluate 0.3 M NaCl with 2.5 vols of ethanol.

(6) Fractionation of RNA by Sucrose Gradient Centrifugation

Poly A+ RNA was purified on a 5-30% linear sucrose gradient in 0.1 M KCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA and 0.2% (w/v) Na-Dod SO_4. Centrifugation was at 20°C for 16 hours at 115,000 g (26,000 rpm, SW41 rotor). The RNA samples in 1 mM EDTA and 0.2% (w/v) Na Dod SO_4 were heated for several minutes at 65°C and quickly cooled on ice before application of a sample (50-150 µl) to the gradient, and centrifugation.

Poly A+ RNA was also purified on a 10 Vs 32% isokinetic sucrose gradient in 10 mM HEPES, pH 7.5, 1 mM EDTA and centrifuged at 2°C for 23 hrs at 270,000 g (40,000 rpm, SW41 rotor). The gradient was constructed by mixing 10% sucrose into a constant volume of 12.1 ml of 32% sucrose for the SW41 tubes.

The linear gradients were fractionated by upward displacement on an ISCO gradient monitor or as below. The isokinetic gradients were fractionated by pumping from the bottom of the centrifuge tube after insertion of a capillary to the bottom. The gradients were collected in approximately 30 equal fractions, NaCl added to 0.3 M, precipitated with 2.5 vols of ethanol, washed with 80% (v/v) ethanol, dried and redissolved in 20-50 µl of 5 mM HEPES pH 7.5. A 1 µl aliquot of the fractions was used in the translation assay.

(7) Messenger RNA translation in Vitro

The RNA was translated with the mRNA dependent rabbit reticulocyte lysate essentially as described by Pelham and Jackson (1976). Samples were translated in a final volume of 12.5 µl or multiples of this volume containing 5 µl of the lysate, up to 2.0 µg of RNA and either 3 µCi of L-(^{3}H) leucine (135 Ci/mmol, Amersham) or 11.5 µCi of (^{35}S) methionine (1400 Ci/mmol, Amersham) plus a mixture of the remaining 19 amino acids, each at 100 µM concentration. The translation mixture also contained 10 mM HEPES pH 7.5, 10 mM creatine phosphate, 80 mM KCl, 0.3 mM spermidine tetrachloride, and 0.4 mM ATP for one lysate. Another lysate differed, its optimum requiring 0.2 mM spermidine tetrachloride, 0.1 mM $MgCl_2$. Creatine phosphokinase (50 µg/mL) and hemin (100 µM, Sigma) were added to the lysate before micrococcal nuclease treatment as described by Pelham and Jackson (1976). Translations were carried out for 60 minutes at 37^{o}C. Incorporation into hot trichloroacetic acid precipitable material was assayed by spotting 1-2 µL aliquots onto 3 MM filter papers (Whatman) as described by Mans and Novelli (1961).

(8) Analysis of RNA by Gel electrophoresis

Agarose gel electrophoresis was performed with glyoxal denatured nucleic acid as described by Carmichael and McMaster (1980). The gel was run with, and in, 10 mM Na_2PO_4 pH 7.0, and the buffer was recirculated to prevent pH changes, as glyoxal is removed from nucleic acid at pH 8.0 or higher. Before use the glyoxal (6 M, 40% technical grade BDH) was deionized with a mixed-bed ion-exchange resin (BDH MB-1) until conductivity was below 5 µMho. The nucleic

acid, typically 5 µg/23.0 µl reaction mixture is incubated in 1 M glyoxal, 50% (v/v) DMSO, 10 mM sodium phosphate buffer pH 7.0 at 50°C for 1 hr. The reaction mixturewas cooled on ice and 6 µl of sample buffer containing 50% (v/v) glycerol, 10 mM sodium phosphate pH 7.0, 0.5% bromophenol blue is added. The samples were usually run on a 1.4% agarose gel. The gel was placed in contact with a nitrocellulose filter (Schleicher and Schüll 0.1 µM, as described by Thomas (1980) equilibrated in 20 xSSC) immediately and the RNA transferred overnight. The transfer solution (20 x SSC) is taken up to the gel by wicks. The nitrocellulose filter was then air dried and baked at 80°C for 2 hrs to immobilize the RNA. The filter was then ready for hybridization with labelled probes.

(9) Immunoprecipitation of Translation Products

Specific antigen-antibody complexes were precipitated with Staphylococcus aureus (Pansorbin, Calbiochem) as described by Kessler (1975).

Aliquots (10-20 µl) of lysate were diluted into about 100 µl of NET buffer (0.15 M NaCl, 50 mM Tris-HCl, 5 mM EDTA, pH 7.4 and 0.02% (w/v) NaN_3) containing in addition 0.05% Nonidet P-40 (NP40), 1 mM phenylmethanesulfonyl fluoride (PMSF) and centrifuged for 10 min at 27000 g (15,000 rpm SS34 rotor) to remove aggregated material. Antisera used were rabbit Immunoglobulin G fractions raised against human IgE, IgM, λ light chain at 10 mg/ml supplied by DAKO (Copenhagen, Denmark), rabbit anti serum raised against IgE (Miles), and Sheep anti serum raised against IgE Fc region (Pharmacia). Immunoprecipitation was carried out by incubation with 0.2-1.0 µL of

the appropriate antibody for 30-60 minutes at 0°C followed by the addition of 10 µL of the S. aureus suspension and incubation for 5-15 minutes. The S. aureus/immune complex was collected by centrifugation at 12,000 g for 30 seconds in an Eppendorf Centrifuge. The supernatants in some cases were retained for further immunoprecipitations, as above. The S. aureus/immune complex was washed in 100 µl of NET buffer 3 times, the polypeptide chain was liberated by heating (100°C) the S. aureus/immune complex in 20 µl of protein electrophoresis loading buffer (Laemmli 1970) (63 mM Tris-HCl pH 6.8, 10% glycerol (v/v), 2% SDS (w/v) 5% 2-mercaptoethanol (v/v), and 0.001% bromophenol blue), for 5 minutes followed by removal of the S. aureus by centrifugation for 30 seconds in an Eppendorf centrifuge.

Protein Gel Electrophoresis

Analysis of protein, and immunoprecipitates was by electrophoresis on 10 or 12% polyacrylamide gels as described by Laemmli (1970). Electrophoresis was typically for 2-3 hrs at 25-35 mA in approximately 1 mm thick gels. After electrophoresis, the gels were processed in several ways.

a) Gels were fixed and prepared for fluorography as described by Bonner and Laskey (1974). The gels were fixed in 50% (w/v) Cl_3CCOOH and dehydrated in 2 changes of DMSO 20 gel volumes for 30 minutes each, and then equilibrated with 4 gel volumes of 2,5-diphenyl-

oxazole (PPO) in DMSO 20% (w/v) for 3 hours. The scintillant was precipitated in the gel by equilibration with 20 gel volumes of water for 1 hour.

b) Gels were fixed in 10% Cl_3CCOOH (w/v), 10% CH_3COOH (v/v), 30% CH_3OH (v/v) if they were to be left overnight, or placed directly in 3 gel volumes of EN^{3}HANCE (New England Nuclear) and shaken for 1 hour. The scintillant was then precipitated in the gel by the addition of 20 gel volumes of cold water and equilibrated for 1 hr.

c) To look at total proteins, gels were fixed and stained in a solution of 25% isopropyl alcohol (v/v), 10% CH_3COOH (v/v) and 0.5 g/L Coomassie Blue G250 (Sigma) overnight and destained in 10% isopropyl alcohol (v/v), 10% CH_3COOH (v/v) followed by 10% CH_3COOH (v/v). (Fairbanks et al., 1971).

Gels processed as above were dried down on Whatmann 3 MM filter paper under vacuum at temperatures of 80°C - 100°C. For visualization of radiolabelled material the gels were exposed to Kodak X-OMat or Fuji RX Xray film at -70°C.

d) Gels were also electrophoretically transferred to nitrocellulose filters using a modified Bio-Rad Trans Blot apparatus as described by Towbin et al., (1979). The gel after electrophoresis was laid on a scouring pad (Scotch-Brite) which was supported by a stiff perspex grid. The nitrocellulose filter 0.1 µm (Schleicher and Schüll) for transfer was equilibrated in the transfer buffer (25 mM Tris, 192 mM glycine, and 20% CH_3OH (v/v) pH 8.3) for 15 minutes and then overlayered onto the gel; any bubbles were removed by smoothing out the filter on the gel. The second scouring pad was then placed on top followed by the second stiff perspex grid. This sandwich was then slid into the slots of the transfer apparatus

containing the transfer buffer. The transfers were typically overnight at ∿20 volts ∿120 mA. The filter containing the bound proteins was stained in amido black (0.1% amido black (w/v), 45% CH_3OH (v/v), 10% CH_3COOH (v/v)) for 5-10 minutes and destained with several washes of 45% CH_3OH, 10% CH_3COOH (v/v); initial use of destaining with 90% CH_3OH, 2% CH_3COOH (Towbin et al., 1979) led to distortion of the nitrocellulose filters. The filters after staining and destaining were equilibrated with PBS, then photographed using a red filter to improve contrast. These nitrocellulose filters were either used immediately for immunological detection of IgE as described later, or air dried and stored.

Iodination of proteins

The method used for all iodinations made use of the water-insoluble Iodogen and ^{125}I as Na I (100 mCi/ml Amersham) (Fraker and Speck, 1978). A stock solution of Iodogen of 1 mg/ml was made in CH_2Cl_2 in a glass tube. This was diluted 10 fold to give the working solution, and 25 µl was measured into 1.5 ml Eppendorf tubes and evaporated completely. Proteins were iodinated at the level of 1 mCi of ^{125}I/66 µg of protein, this being for IgG a molar ratio of IgG/$I^{\ominus}$ of 0.9. The protein and ^{125}I were incubated in 25 µl at room temperature for 10 minutes, with mixing at 1 minute intervals. The iodinated protein was separated from unincorporated iodide by a Sephadex G50 column (10 mm x 150 mm) in PBS. Typically, incorporation was greater than 50% as determined by Cl_3CCOOH precipitable counts. Aliquots of the iodide free labelled protein were counted to determine the level of activity for subsequent

experiments.

(12) Immunological Detection of Proteins in Nitrocellulose

The electrophoretic blots after staining with amido black were soaked in 3% bovine serum albumin (BSA) in PBS, sealed into a plastic bag, for 1 hour at 40°C to block any remaining protein binding sites. This preincubation medium was squeezed out and fresh 3% BSA in PBS now containing ^{125}I-labelled IgG at 10^6 cpm/ml (affinity purified rabbit anti IgE PS ε chain, ε 2 domain; gift from Dr. Isizaka), or Peroxidase conjugated IgG at a 1 in 300 dilution (affinity purified Goat anti-human IgE ε chain specific, TAGO, Inc) was added. Typically 10 mL of 3% BSA, PBS with antibodies was added to a 20 cm by 20 cm nitrocellulose filter, they were incubated at room temperature for 1-2 hours or at 0°C overnight. The filters after incubation were washed in PBS containing 0.05% NP40 with up to six changes with total times from 1 hr - 8 hr.

The blots exposed to the radiolabelled antibodies were dried and exposed at -70°C to Fuji RX Xray film with intensifying screens (Ilford). The blots exposed to the peroxidase conjugated antibodies were developed using a combination of methods (Towbin et al., 1979, and Bos et al., 1981). The colour reaction was developed by incubation in a solution of 0.1 mg/ml 3,3',5,5' tetramethyl benzidine (TMB, Miles), 0.0044% H_2O_2 and 20 mM Tris-HCl pH 7.5 for times up to 2 hrs. This solution was prepared freshly from stock solutions of 10 mg/ml TMB in DMSO stored in the dark, and 30% H_2O_2. The use of lower pH, e.g. pH 6.0, resulted in production of a soluble blue colour instead of the brown precipitate.

(13) Enzyme Linked Immuno Sorbent Assay (ELISA)

An enzyme linked assay was developed for extracts from E. coli expressing Ig ε antigenic determinants, based on the double antibody sandwich ELISA described by Voller et al., (1979), using affinity purified goat anti-Human IgE ε chain specific antibody unconjugated and Horseradish peroxidase conjugated (TAGO Inc). Microtiter plates (Nunc-Immuno Plate I flat bottomed, Gibco) were coated with unconjugated antibodies or control antibodies at 100 µl/well, 3 µg IgG/mL in coating buffer (1.59 gm anhydrous Na_2CO_3, 2.93 gm $NaHCO_3$ in 1 litre of water pH 9.6) overnight at 4°C. The contents of the wells were then shaken out, washed with PBS/0.05% NP40 twice, and 100 µL of PBS, 0.5% casein (Hammarsten BDH) was added to each well and incubated, either overnight at 4°C or at room temperature for 30 minutes, to attempt to block any remaining protein binding sites. These plates were shaken out and washed with PBS/0.05% NP40 twice. These plates were then ready for doubling dilutions of samples. The first row was left without any sample and the second row had 200 µL of sample in the assay buffer. The rest of the plate had 100 µL of assay buffer, and 100 µL of the sample row was taken up and doubling dilutions made across the plate to row 12 where the excess 100 µL was discarded. The assay buffer finally selected which prevented the problems with plate binding of the bacterial expressed Ig ε, was 3% BSA (w/v), 0.5% casein (w/v), 2.25 M urea, 10 mM EDTA, 5 mM NH_4Ac pH 4.5 and 5% E. coli extract (v/v). The E. coli extract was prepared in the same way as for the samples thus maintaining the E. coli proteins at essentially the same level such that only the expressed products from

the inserted gene segment were diluted. This E. coli extract also diminished plate binding by the Ig ε (Chapter 7). The E. coli extract was prepared by lysis of 6.0×10^9 cells (5 mls of culture at an Absorbance 600 nm of 1.5), in 1 ml of lysis buffer (9 M urea, 0.2% SDS (w/v), 0.5% NP40 (v/v), 10 mM EDTA, 2 mM PMSF, 5 mM N-ethylmaleimide (NEM), 5 mM NH_4Ac pH 4.5). The samples were stored at -70°C, followed by lysis by heating at 100°C for 5 minutes. The samples for assay were prepared in the same way and diluted to 5% (v/v) to give the assay buffer as described above for the E. coli extract.

After doubling dilution of the samples for testing, including a standard on each plate, the plate was incubated for 2-4 hr at room temperature or overnight at 4°C. The plate after its incubation with the test samples was washed 4 times with PBS/0.05% NP40, followed by incubation with 100 µL of peroxidase conjugated affinity purified goat anti-human IgE ε chain specific, diluted 1:300 in 0.5% casein (w/v) PBS for 1-2 hrs at room temperature. After this incubation, the plate was washed 4 times with PBS/0.05% NP40 and shaken free of excess wash buffer, and 100 µL of the substrate for the peroxidase added (0.1 mg/mL TMB, 0.0044% H_2O_2, 0.1 M CH_3COONa/Citric acid buffer pH 6.0). The blue colour was allowed to develop for 60-80 minutes and the reaction terminated by addition of 25 µL of 2.5M H_2SO_4. The absorbance was measured on a microtiter plate reader at 450 nm with the reference absorbance set at 630 nm. The absorbance was plotted against the dilution, the dilution point for various samples was determined at a defined absorbance value of 0.8-0.64 with reference to the standard.

(14) Synthesis of oligonucleotides

The initial four oligonucleotide primers were provided by the Chemistry Department of G. D. Searle. Under their direction, we synthesised two oligonucleotides, primers P6 and P8 (see Chapter 4). The extension of the latter two primers' partial products was carried out by the Chemistry Department. All these oligonucleotides were synthesised using solution chemistry (Houghton et al., 1980a) but the oligodeoxynucleotides used in construction of the probe for screening colonies were made by the Chemistry Department using solid phase techniques (Miyoshi et al., 1980).

(15) 5'-end labelling of Oligodeoxynucleotides (Houghton et al., 1980a)

The oligonucleotides were 5' labelled using (γ^{32}P) ATP and T4 polynucleotide kinase. This kinasing was done in as small a volume as convenient. Typically 250 µCi of (γ^{32}P) ATP (specific activity >5000 Ci/mmol) was dried down in vacuo in a 15 ml siliconized and autoclaved corex tube. The residue was taken up in kinase buffer (25 mM Tris-HCl pH 9, 5 mM $MgCl_2$, 50 µM Na_2EDTA, 50 µM spermidine and 1 mM dithiothreitol (DTT) and 30 pmoles of the oligodeoxynucleotide primer was added. The T4 polynucleotide kinase (PNK), (1 µL, New England Biolabs, 2-1.5 x 10^3 u/ml) was added to the mix (9 µL) which was then transferred to a siliconized, autoclaved Eppendorf and incubated at 37°C for 60 minutes. After the incubation the reaction was diluted (150 µL) and an aliquot spotted onto a polyethylenimine (PEI) cellulose thin layer chromatography plate (BDH) and developed using 0.75 M K_2HPO_4, pH 3.5,

followed by detection with Xray film Kodak X Omat or Fuji RX plus an intensifying screen (Ilford). The labelled oligonucleotide remained bound to the origin and the ($\gamma^{32}P$) ATP migrated up the plate and so the amount of ($\gamma^{32}P$) ATP incorporated could be estimated (Maxam and Gilbert 1980). The remaining mix was diluted (200 µL) and NaDod SO_4 and EDTA were added to 0.1% (w/v) and 10 mM respectively. This solution was extracted twice with 2 volumes of water saturated phenol/$CHCl_3$ ($^1/1$, v/v), followed by 2 extractions with 2 volumes of $CHCl_3$. The aqueous phase was then precipitated with 3 µg of DNA-free tRNA (Yeast, Calobiochem) 10 µL of 4 M NaCl and 3 volumes of ethanol at -20°C overnight. The labelled oligonucleotide and tRNA were collected by centrifugation 10,000 g (10,000 rpm, Sorvall HB4), washed with absolute ethanol, dried under vacuum and resuspended in sterile water as required.

(16) Sequencing of synthetic oligodeoxynucleotides

The 'wandering spot' procedure of Brownlee and Sanger was used as described (Tu et al., 1976). The oligodeoxynucleotides (primers) were labelled with (^{32}P) at the 5' end by kinasing as described previously and electrophoresed on a 15% polyacrylamide, 7 M urea gel. The primer was located by autoradiography, the band cut out and the primer eluted by crushing the gel slice and resuspension in 0.1 M CH_3COONH_4, 5 mM Mg $(CH_3COO)_2$, 0.02% NaDod SO_4 and 20 µM EDTA pH 7.0 with shaking overnight. The polyacrylamide gel was removed by filtration and the primer precipitated, after adding NaCl to 0.2 M and t-RNA to 10 µg/ml, with 2.5 volumes of ethanol followed by an overnight incubation at -20°C. The precipitate was collected by

centrifugation at 10,000 g for 15 minutes (10,000 rpm Sorvall HB4).

The dried primer was taken up in 30 µL of 5 mM Tris-HCl pH 8.3, 5 mM KH_2PO_4, 2 mM $MgCl_2$ and 3 µg/ml tRNA and divided into 10 aliquots, to each of which was added one fifth volume of snake venom phosphodiesterase (Sigma) at the following concentrations 0, 5, 10, 50, 100, 125, 150, 250, 500 µg/mL. After incubation at 37°C for 15 minutes the reaction was stopped by the addition of EDTA to 5 mM pH 7.0 and 0.5 µL aliquots from each reaction mixture were chromatographed on a PEI cellulose thin layer plate (BDH) in 0.75 M K_2HPO_4, pH 3.5 (Maxam and Gilbert 1980). These were autoradiographed for two hours, and the extent of the digestion was indicated by the degree to which the radioactivity had travelled up the plate. Reaction mixtures corresponding to digestion patterns from just before any sign of digestion to a complete digestion were pooled, dried on a rotary evaporator and reconstituted in 4 µL of water containing 0.1% acid fuchsin (Sigma Ltd) and spotted 8 cms from the end of a 4 cm x 1 m cellulose acetate strip (Schleicher and Schüll) pre-equilibrated in 5% acetic acid, 7 M urea adjusted to pH 3.5 with pyridine. Electrophoresis was carried out in this buffer under white spirit at 5,000 volts for 40 minutes. The strips were washed in ether to remove the white spirit. The oligonucleotides were located using a radiation detector and transferred onto a DEAE cellulose plate. The strip containing the radiolabelled oligonucleotides was laid along a glass rod on top of a wet 3 MM (Whatman) filter paper, the edges of the strip were held down by more wet 3 MM paper held in place by glass plates. The DEAE cellulose plate was laid on top of the cellulose acetate strip

and held in contact by a glass plate. The oligonucleotides were eluted from the cellulose acetate to the DEAE cellulose concentrating it as a tight band. The excess urea was washed off the TLC plate with water prior to the chromatography in the second dimension. The second dimension was developed by 4% homomix (Brownlee and Sanger 1969, prepared as described below and provided by Dr. J. M. B. Warwick) at 50°C. The plate after 16 hours with the solvent front at the top of the plate was left to dry, and was autoradiographed with an intensifying screen using Kodak X Omat Xray film.

The 4% homomixture was prepared by dissolving 10 gms of yeast RNA in 100 mls of 1 N KOH and hydrolysing it for 15 minutes at room temperature. The pH was adjusted to 7.5 with concentrated HCl, followed by dialysis versus water for 2-4 hours, and subsequent addition of urea and dilution to 200 mls to produce a 5% homomix in 7 M urea.

(17) Specific oligodeoxynucleotide 'primer' directed synthesis of cDNAs using AMV reverse transcriptase on poly A+ RNA

The method used was similar to that of Houghton et al., (1980a). The poly A+ RNA was typically preincubated with the primer to be used in a 10-20 µL volume containing 1.5 µM primer, 0.2 M KCl and 300 µg/ml poly A+ RNA. After preincubation for 1 hr at 25°C, about 5 volumes of the reverse transcriptase mix was added. The final mixture contained, 0.3 µM primer; 60 µg/mL poly A+ RNA; 60 mM KCl; 0.5 mM dATP, dCTP, dGTP and dTTP; 5 mM DTT; 50 mM Tris-HCl pH 8.3; 4 mM $MgCl_2$; 0.01% Triton X-100 (v/v); 50 µg/ml actinomycin D; 20 u/mL rat

liver ribonuclease inhibitor (Searle Research Products); and 240-200 u/mL AMV reverse transcriptase (Beard Life Science). It was incubated at 37°C for 1 hr, and the reaction stopped by adding NaDod SO_4 to 1% and EDTA to 10 mM and a final volume of 200 mL. This reaction mixture was extracted first with 2 volumes of water saturated phenol/$CHCl_3$ (1/1), (v/v) and then with 2 volumes of $CHCl_3$. The aqueous phase was removed and DNA precipitated after adding NaCl to 200 mM, 2 µg of DNA free tRNA (Yeast, Calobiochem) followed by 2.5 volumes of ethanol and incubation overnight at -20°C. The primed transcripts were pelleted at 10,000 g (10,000 rpm Sorvall HB4), washed with ethanol and dried under vacuum.

The primed transcripts were analysed by alkaline agarose gel electrophoresis (McDonell et al., 1977). The gels were 1.4 to 1.2% agarose (Sigma type I), 30 mM NaOH, 2 mM EDTA. The primed transcripts were taken up in a 20 µL of 10% ficoll, 0.1% bromocresol green, 30 mM NaOH and 2 mM EDTA. The samples were run on 0.5 cm x 20 cm vertical slab gels at 50 volts overnight. Marker fragments used were PM 2 phage (Boehringer Corp.) digested with Hind III and end labelled with (^{32}P), or ØX 174 digested with Hae III (Biolabs). The primed transcripts were revealed by autoradiography of the wet gel if the transcripts were to be extracted from the gel, or blotted dry on to DE81 paper (Whatman). The wet gels were exposed at 4°C; dried gels were exposed at -70°C using an intensifying screen and Xray film Kodak X Omat or Fuji RX.

The primed transcripts to be extracted were cut from the gel, using an autoradiograph referenced with radioactive ink spots as a

template. The gel slices were extruded through a 21G needle from a 2 mL syringe, washed with 2 to 4 0.5 mL aliquots of 100 mM Hepes pH 7.0, 1 mM EDTA and 0.02% Triton X 100 (v/v) onto the gel slurry. The slurry was then frozen at -70°C, thawed and shaken gently overnight at room temperature. The agarose was filtered off by spinning (1,500 g, 4,000 rpm Sorvall HB4) through a 5 mL syringe packed from the tip with a GF/C, and a GF/B (Whatman) filter and finally sterile siliconized glass wool to form a tightly packed plug of 2 mL volume, and washed with 0.5 mls of the extraction buffer described above. To the filtered extract, 40 µg of sonicated calf thymus DNA was added and the mixture extracted with 2 volumes of water saturated phenol/ $CHCl_3$ (1/1, v/v), and then with 2 volumes of $CHCl_3$. The aqueous layer was made 0.2 M with respect to NaCl, and DNA was precipitated with 3 volumes of ethanol at -20°C overnight. The labelled DNA was obtained by centrifugation at 10,000 g for 30 minutes (10,000 rpm Sorvall HB4), washed with alcohol and dried in vacuum. The DNA pellet was ready for sequencing, or use as a hybridization probe.

(18) Electrophoresis of DNA

DNA was usually analysed by electrophoresis in agarose or polyacrylamide gels. DNA fragments ≥500 bases were analysed by agarose gels and fragments ≤1000 bases by polyacrylamide gels. Agarose gels were 1.4% - 1.2% agarose (DNA fragments 10,000 - 200 base pairs) in either TAE (40 mM Tris, 5 mM sodium acetate and 1 mM EDTA pH 7.7 McDonell et al., 1977) or TBE (50 mM Tris, 50 mM boric acid, 1 mM EDTA pH 8.3 Peacock and Dingman 1968), TAE was initially used for electrophoresis of DNA fragments and supercoiled

plasmids as TBE gives anomalous migratory properties to supercoiled plasmids. Polyacrylamide gels prepared from a stock solution of 29% acrylamide and 1% methylene bisacrylamide (w/v) (15% gel, 1-50 nucleotides; 8% gel, 25-200 nucleotides; 6% gel 200-1,000 nucleotides), were run in TBE. The DNA samples were loaded in volumes up to 30 µl depending on sample well size using 8% Ficoll, 0.025% of bromophenol blue and xylene cyanol with either TBE or TAE buffer. In some cases restriction digests were loaded directly on to agarose gels after adding NaDod SO_4 to 0.1%.

The DNA in these gels was visualized by staining in ethidium bromide at 2-5µg/mL and allowing the gel to equilibrate for ~80 minutes. The DNA was seen as fluorescent bands under ultraviolet light. Radiolabelled DNA fragments of sufficient activity were visualized by autoradiography using an intensifying screen and Xray film Fuji RX exposed at 4°C to reduce diffusion of bands.

Transfer of DNA fragments from agarose gels for hybridization was done to nitrocellulose filters (Scheicher and Schüll) using the method of Southern (1979). The DNA agarose gel, after staining with ethidium bromide for photography, was washed in 250 mL of 0.25 N HCl 2 times for a total time of 10 minutes. This results in partial depurination of the DNA (for a 1 cm thick gel) causing fragmentation on alkaline treatment, Wahl et al., 1979, this gives a more even transfer efficiency for all sizes of DNA. This procedure was invaluable for supercoiled plasmid DNA allowing its complete denaturation for transfers. The gel was next washed in 250 mL of 0.5 N NaOH and 1.5 M NaCl 2 times for a total time of 30 minutes,

to cause denaturation and cleavage at depurinated bases. The gel was rinsed in water, followed by a 250 mL wash in 2 M NaCl and 1 M Tris-HCl pH 7.0 two times for a total of 30 minutes. The transfer apparatus consisted of a reservoir of 20 x SSC with filter wicks running up to a platform of 3 mm filter paper saturated with 20 x SSC onto which the gel was placed. The gel was then masked round the edges with parafilm and the nitrocellulose filter, prewetted in water and equilibrated in 20 x SSC was placed on the gel surface, smoothing out any air bubbles. The transfer of DNA was facilitated by placing a large stack of lightly weighted down absorbent paper towels over the nitrocellulose filter and left overnight. The nitrocellulose filter was then washed in 2 x SSC, air dried and heated at 80^{o}C for 2 hr.

Recovery of DNA fragments from Agarose and Acrylamide Gels

Native DNA fragments separated by gel electrophoresis were recovered by electrophoresis onto DEAE cellulose powder or paper (Winberg and Hammarskjold 1980; Dretzen et al., 1981).

The elution into DEAE cellulose powder was done with a 2 ml disposable syringe containing 0.25 mls of sterile DE52 (Whatman), loaded into a tube gel electrophoresis tank. The tank was filled with TBE, the buffer running through the DE52 in the syringe to the bottom electrophoresis tank. The gel slice containing the DNA was placed in a 1 ml Gilson pipette tip with the end cut off to widen the hole without letting the gel slice fall through. The Gilson tip with the gel slice was introduced into the electrophoresis tank with

out trapping any air bubbles and the gel slice pushed down to the tip, which was then placed inside the 2 mL syringe and weighted down with a small magnetic stirrer. This was then electrophoresed at 200 volts for 1-8 hrs depending on the size of the DNA fragment and the gel percentage. This method was also used for the extraction from a 15% polyacrylamide gel in 7 M urea, TBE, of the single stranded 22 oligonucleotide probe used to screen colonies (Chapter 5).

The elution into DEAE paper, DE81 (Whatman) was done without removal of a gel slice containing the DNA. The agarose below the DNA band was cut open and a piece of pretreated DE81 paper (see below) was introduced, the gel returned to the electrophoresis chamber and electrophoresis continued until the DNA had electrophoresed into and bound to the DE81 paper. The DE81 paper is pretreated by soaking for several hours in 2.5 M NaCl, washed in water and stored in 1 mM EDTA at $4^{o}C$.

The DEAE cellulose powder or paper containing the bound DNA was washed several times with 0.1 M CH_3COONH_4, 5 mM Mg $(CH_3COO)_2$, 0.02% $NaDodSO_4$ (w/v) and 20 µM EDTA pH 7.0. The DNA was eluted with the same buffer used for the wash but made 1.1 M with respect to NaCl. The DNA was precipitated by addition of 3 volumes of ethanol to the eluted DNA, followed by incubation overnight at $-20^{o}C$ and collected by centrifugation at 10,000 g for 30 minutes (10,000 rpm Sorvall HB4). The pellets were washed in 70% ethanol twice and vacuum dried.

Molecular Cloning of 266 BL poly A+ RNA

(20) Synthesis of double stranded cDNA

The protocol was derived from those used by Buell et al., (1978)

and Monahan et al., (1976) for first strand synthesis (1 s cDNA) and Emtage et al., (1979) for second strand synthesis (2 s cDNA). Synthesis of the first strand was carried out for 60 minutes at 37°C in a 100 µL reaction containing 20 µCi of (γ^{32}P) dCTP (Amersham, specific activity 200-400 Ci/mmol); 50 mM Tris-HCl, pH 8.3; 0.5 mM dATP, dGTP, and dTTP; 10 mM $MgCl_2$; 0.01% Triton X-100 (v/v); 50 µg/mL actinomycin D (Calobiochem); 50 mM KCl; 10 mM DTT; 20 µg/mL oligo (dT) 12-15 (PL biochemicals); 20 u/mL rat liver ribonuclease inhibitor (Searle Research Products) and 240-200 u/mL AMV reverse transcriptase (Beard Life Sciences). RNA was used up to the level of 30 µg/mL. After incubation, the reaction was stopped by adding Na Dod SO_4 to 1% (w/v) and EDTA to 10 mM and the volume was made up to 200 µl. The stopped reaction mixture was then extracted with 2 volumes of water saturated phenol/$CHCl_3$ (1/2, v/v), followed by 2 vols of $CHCl_3$. The aqueous phase was removed, made 200 mM with respect to NaCl, precipitated with 2 µg of DNA free t RNA (Yeast Calobiochem) and 2.5 vols of ethanol at -20°C overnight. The lscDNA was collected by centrifugation at 10,000 g for 30 minutes (10,000 rpm Sorvall HB4), the supernatant discarded and the pellet dried in vacuo.

RNA was removed from the lscDNA by alkaline treatment. The lscDNA pellet was reconstituted into 100 µL of 0.2% Na Dod SO_4, 5 mM EDTA and 0.1 M NaOH followed by an incubation for 20 minutes at 70°C. The incubation was terminated by addition of 1 µL of 1% phenol red (w/v), 7.5 µMoles of Tris-HCl pH 8.0, and 10 µMoles of CH_3COOH, followed by chromatography on a Sephadex G150 column (0.7 cm x 34 cm) in 150 mM NaCl 10 mM Tris-HCl pH 7.5 and 0.1% Na Dod SO_4. The

excluded peak was collected, precipitated without added tRNA as above and washed with 70% ethanol.

The dried 1scDNA pellet from the G150 was taken up in 100 µL of 5 mM dA, dC, dG and dT triphosphates; and 1000 u/mL of AMV reverse transcriptase (J. Beard Life Sciences). This mixture was overlayered with paraffin and incubated for 3 hrs at 45°C. After incubation most of the paraffinwas removed, $NaDodSO_4$ was added to 1% w/v and EDTA to 10 mM and the mixture extracted with 2 volumes of $CHCl_3$ to remove residual paraffin. This mixture was then extracted with 2 volumes of water saturated phenol/$CHCl_3$ (1/1, v/v), followed by two extractions with 2 volumes of $CHCl_3$. The aqueous phase was then made 20% (v/v) in glycerol, and 0.01% (w/v) in phenol red and chromatographed on a Sephadex G50 column. The excluded peak was pooled made 200 mM with respect to NaCl, and the DNA precipitated by the addition of 1.5 µg of tRNA followed by 2.5 volumes of ethanol and incubation overnight at -20°C. The double stranded cDNA was then collected by centrifugation and washed 2 times with 70% ethanol.

The removal of the single stranded regions including the hair pin from the double stranded cDNA (2scDNA) was done by taking up the double stranded cDNA in 150 mM NaCl, 25 mM $NaCH_3COO$ pH 4.6, 1 mM $ZnSO_4$ and 15 units/mL of SI nuclease followed by incubation at 37°C for 30 minutes. The incubation was terminated by adding $NaDodSO_4$ to 0.2% (w/v), EDTA to 20 mM and Tris;HCl pH 8.6 to 50 mM in a final total volume of 300 µl. This terminated reaction mixture was then extracted with 2 volumes of water saturated phenol/$CHCl_3$ (1/1, v/v) followed by 2 extractions with 2 volumes of $CHCl_3$. The aqueous

phase was made 200 mM NaCl and DNA precipitated with 2.5 volumes of ethanol. The 2scDNA was collected by centrifugation at 10,000 g for 30 minutes and dried under vacuum. This DNA was fractionated by gel electrophoresis followed by polymerase I treatment as described for method (3) (see below) after SI treatment. The DNA was also linkered as described below.

(21) Synthesis of double stranded cDNA

The protocol was derived from those used by Wickens et al (1978) and Gough et al., (1980). Synthesis of the first strand was carried out for 60 minutes at 37°C in a 50 µL reaction, or multiples containing 20 µCi of (α^{32}P) dTTP (Amersham 200-400 Ci/mmol); 50 mM Tris-HCl, pH 8.3; 0.5 mM dATP, dGTP, and dCTP; 150 µM dTTP; 20 mM KCl; 5 mM DTT; 4 mM $MgCl_2$; 25 µg/mL oligo (dT) 12-15 (PL Biochemicals) and 200 u/mL of AMV reverse transcriptase (J. Beard Life Sciences). This mixture was sampled before and after incubation to monitor incorporation, 1 µL aliquots precipitated on to 3 MM filters, washed in 5% CCl_3COOH (w/v) and 3% sodium pyrophosphate (w/v) 10 times were dried prior to liquid scintillation counting. The mixture was then boiled for 3 minutes, and the pH and salt concentration were adjusted by adding an equal volume of a solution containing 90 mM Hepes K^+ pH 7.2; 2.5 mM DTT; 0.6 mM dATP, dGTP, dCTP and dTTP; 134 mM KCl and 17 u/ml of E. coli DNA polymerase I.

(22) Synthesis of double stranded cDNA

The final method was almost identical to that described by Gough et al., (1980). Synthesis of the first strand was carried out for 45 minutes at 42°C typically in 50 µl or multiples thereof

containing 20 µCi of (α^{32}P) dTTP (Amersham 200-4PP Ci/mmol); 50 mM Tris-HCl, pH 8.3; 0.5 mM dATP, dCTP, and dGTP; 150 µM dTTP; 6.0 mM Mg $(CH_3COO)_2$; 5 mM DTT; 20 µg/mL (dT) 12-15 (PL Biochemicals); and 200 u/mL of reverse transcriptase as above. The reaction was monitored as above, boiled for 60 seconds, and the pH and salt concentrations were adjusted by adding 6.25 µL of 0.4 M Hepes K^+, pH 7.2 and 0.5 M KCl to 45 µl of first-strand reaction mixture. The second strand synthesis was carried out at 15°C for 3 hrs with 10 units of E. coli DNA polymerase I. The reaction was stopped by extraction with 2 volumes of water saturated phenol/$CHCl_3$ (1/1, v/v) followed by 2 extractions with 2 volumes of $CHCl_3$. The cDNA was then precipitated as above and vacuum dried.

For SI nuclease treatment, the double stranded cDNA was dissolved in 200 µL containing 0.5 M NaCl, 50 mM $NaCH_3COO$, pH 4.6, 2 mM $ZnSO_4$, and 250 u/mL of SI nuclease (BRL). The mixture was incubated at 37°C for 30 minutes. The digest was made 0.1 M with respect to Tris-HCl, pH 9.0 and 10 mM to EDTA, and extracted with water saturated phenol/$CHCl_3$ (1/1, v/v) followed by 2 extractions with 2 vols of $CHCl_3$.

The SI-treated double stranded cDNA was fractionated on an 8 mm x 360 mm column of 100-200 mesh Bio-Gel A150 M agarose (Bio-Rad) in 0.15 M NaCl and 2 mM EDTA, pH 8.0. (The column was pre run with 70 µg of tRNA (E. coli) to reduce nonspecific binding.) Ten drop fractions were collected (~ 340 µL), and selected fractions were pooled and $Mg(CH_3COO)_2$ added to 10 mM. The cDNA was precipitated with ethanol for several hours at -70°C, and recovered by

centrifugation in polyallomer tubes for 2 hrs at 40,000 rpm (Beckman SW41 rotor) and reconstituted from the washed, vacuum dried pellet in 20 µL of 10 mM Tris-HCl, pH 7.5.

The fractionated, SI-treated double stranded cDNA was then incubated in a 50 µL reaction with 50 mM Tris-HCl, pH 7.5; 10 mM $MgCl_2$; 1 mM 2-mercaptoethanol; 200 µM dATP, dCTP, dGTP and dTTP and 500 u/mL E. coli DNA polymerase I at 15°C for 20 minutes. The reaction mixture was adjusted to 0.2% with respect to $NaDOdSO_4$ and 20 mM to EDTA, extracted with 2 volumes of water saturated phenol/ $CHCl_3$ (1/1, v/v) and twice extracted with 2 volumes of $CHCl_3$. The interface was re-extracted with 5 µL of 4 M, CH_3COONH_4, and 10 µL of water. The fractionated double stranded cDNA was precipitated with 150 µL of ethanol.

(23) Linker ligation to (linkering) the double stranded cDNA

Linkers constructed to the Hind III restriction endonuclease site (Collaborative Research) were kinased (see labelling of primers) to enable their ligation. The linkers were reconstituted to a 5' end concentration of 150 µM. 5 µL of these linkers were incubated at 37°C for 30 minutes in a 50 µL mix containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 250 µM ATP, 50 µg/ml gelatin, 200 u/ml T4 polynucleotide kinase (PL biochemicals) and 100 µCi ($\gamma^{32}P$) ATP (> 5 Ci/µmol). The linkers were rekinased by the addition of 10 u of T4 polynucleotide kinase in 10 µL of 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 4 mM ATP and incubation for a further 30 minutes at 37°C. To the kinased linkers $NaDodSO_4$ was added to 0.2% and EDTA to 5 mM followed by extraction first with

2 volumes of water saturated phenol/$CHCl_3$ (1/1, v/v), and then twice with 2 volumes of $CHCl_3$. The linkers were precipitated with tRNA at 10 µg/mL, NaCl at 0.2 M by addition of 2.5 volumes of ethanol followed by incubation at -20°C overnight. The precipitated linker was collected by centrifugation 10,000 g for 15 minutes (10,000 g Sorvall HB4). The precipitate was washed with ethanol, vacuum dried and reconstituted in 50 µL of water to 15 µM.

The kinased linkers were tested for their ability to self ligate and to be digested with restriction endonuclease Hind III. Typically a 10 µL incubation is carried out at 25°C overnight containing 3 µM Hind III linker, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 20 mM DTT, 1 mM ATP, and 4 x 10^4 u/ml T4 DNA ligase (Biolabs). This incubation was split and 5 µL digested with Hind III restriction endonuclease in a 100 µL reaction volume as described by Biolabs using 5 units (15 minute units) of Hind III, at 37°C for 1 hr. The linkers before ligation, after ligation, and after ligation and cutting were analysed on a 6% acrylamide - 0.2% bisacrylamide gel in Tris-borate-EDTA, pH 8.3, running the bromophenol blue 2/3 of the gel length.

The double stranded cDNA was ligated to an excess of linkers: 50-75 fold molar excess of 5' termini linkers to 5' termini of double stranded cDNA were used in a 15 µL incubation containing 6 µM 5' termini Hind III linker, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 20 mM DTT, 1 mM ATP, 1.6 x 10^3 units of T4 DNA ligase (Biolabs) and 120-80 nM 5'termini double stranded cDNA at 25°C overnight. The ligation incubation was then heated at 65°C for 5 minutes and made up to 200 µL for Hind III restriction endonuclease digestion using 30 units of Hind III restriction endonuclease (Biolabs). The linkered

genes were separated from the cut linker by gel filtration on sephadex G150 0.7 cm x 34 cm, with 150 mM NaCl, 10 mM Tris-HCl pH 7.5 and 0.1% $NaDodSO_4$ (w/v), collecting 25 drop fractions. The excluded peak was pooled and precipitated.

(24) Bacterial Cultures

The Escherichia coli (E. coli) used were K12 strains; MRC8 (dap 103 hsd R Met B1 glm 533 upp 1 dap 101 sup E thy A 103 deo rec A1), HB101 (F^-, hsd S20 (r_B^-, M_B^-), rec A13, ara 14, Pro A2, Lac Y1, gal K2, rps L20 (Sm^-), xyl-5, mtl-1, sup E44, λ^- and WT217 (ara D 139 (ara-len) de 7697 (lac I POZY) de 174 gal U gal K hsd R rpsl srl rec A56). All media was autoclaved at 15lbs pressure for 15 minutes and thermolabile supplements were filter sterilized using 0.45 µm nitrocellulose filters (Millipore or Schleicher and Schüll). These strains were usually grown in L broth (1% Bacto-tryptone (Difco), 0.5% yeast extract (Difco), 0.5% NaCl, 0.2% glucose, all (w/v) and 40 µg/mL thymine (Sigma)). For MRC8 this medium was supplemented after autoclaving with fresh 50 µg/mL DL-α, ε-diaminopimelic acid (DAP) and 200 µg/mL N-acetyl-D-glucosamine (NAG) (Sigma Ltd). Strains were also grown on agar plates containing 1.5% (w/v) agar (Difco) and either 2.5% (w/v) nutrient broth No. 2 (Oxoid) or L Broth, supplemented as above for MRC8 by addition of DAP and NAG after autoclaving to the cooled agar (∿ 65°C). The E. coli containing the plasmid pAT 153 or its derivatives were grown as above, but with the medium supplemented after autoclaving with 100-200 µg/mL carbenicillin (Sigma), because all manipulations of inserted genes occurred at the Hind III site and did not affect the β lactamase gene which confers

resistance to carbenicillin. The bacteria were cultured at 37°C with shaking of liquid cultures (∿ 140 rpm). MRC8 was sensitive to shaking on reaching saturation, thus overnight cultures were not shaken or only very slowly (∿ 40 rpm).

The storage of E. coli strain and those carrying plasmids was at -20°C in 50% (v/v) glycerol. The strains to be stored were grown up in L-Broth overnight and the culture made 50% (v/v) glycerol by addition of 750 µL sterile 80% (v/v) glycerol, per 450 µL of bacterial culture. These stocks were streaked out on agar plates before culturing, and with MRC8 tested for sensitivity to the absences of DAP or NAG.

The growth of bacteria for expression studies was done in M9 medium as follows: 1 x M9 salts (0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl and 0.1% NH_4Cl all (w/v)), 0.5% cas amino acids, 1% glucose, 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 1 µg/mL vitamin B1 (BDH, 1 mg/mL filter sterile stock) and 100-200 µg/mL carbenicillin (Sigma Ltd). The bacterial cultures..were grown up overnight in L-Broth and innoculated at the rate of 0.1% to 0.5% (v/v) of the M9 culture. The M9 medium as above contains negligible amounts of tryptophan and to study control of expression it was supplemented at innoculation with tryptophan (100 µg/mL, from 10 mg/mL filter sterile stock) or 3-β-indole acrylic acid (10 µg/mL, from 10-20 mg/mL of ethanol stock) at culture absorbances (600 nm) or 0.2 - 0.3.

Plasmid DNA preparation

(25) Cleared lysate method: (Clewell and Helinski 1969, Piko et al., 1968).

The E. coli containing the required plasmid was streaked out on agar plate with the appropriate medium, grown overnight, a single colony picked out and grown overnight in 20 mL of L Broth (1% Bacto-tryptone (w/v), 0.5% yeast extract (w/v) (Difco), 0.5% NaCl (w/v), 0.2% glucose (w/v), and 40 µg/mL thymine (Sigma)) supplemented with 100 µg/mL carbenicillin (Sigma). This culture was used to innoculate 1L of the same medium and grown overnight. The cells were pelleted, washed with 10 mM Tris-HCl pH 8.0, 1 mM EDTA and repelleted. The cells were resuspended in 6 mL of ice-cold 25% sucrose, 50 mM Tris-HCl pH 8.0, followed by 2 mL of fresh lysozyme (10 mg/mL). This mix was swirled gently on ice for 5 minutes, then 2 mL of 0.4M EDTA pH 8.5 was added and again the mixture was gently swirled on ice for 5 minutes. These protoplasts were lysed by the addition of 10 mL of ice-cold 2% Triton x 100 (v/v), 50 mM Tris-HCl pH 8.0, with gentle swirling on ice for 10 minutes.

The lysed protoplasts were spun at 25,000 g, 4°C, for 60 minutes (18,000 rpm Sorvall SS34). The supernatant was removed leaving behind the bulk of chromosomal DNA. The volume of the supernatant was determined and CsCl added at the rate of 0.95 gm/mL, and ethidium bromide 10 mg/mL at 0.02 mL/mL. This was mixed well and spun in a Ti 50 rotor for 48 hrs at 10°C 126,000 g [av] (Ti50, 40,000 rpm), in half filled centrifuge tubes overlaid with liquid paraffin.

The plasmid DNA was visualised by examination of the tubes in long wave (366 nm) ultra violet light (to prevent extensive nicking of the DNA). The lower plasmid band should be the most prominant band. This was collected by side puncture using a 16 gauge needle and a 1 ml syringe. The ethidium bromide was removed by equal volume extractions with n-butanol and the extracted CsCl solution containing the plasmid DNA was then diluted with 3 volumes of sterile distilled water or 10 mM Tris-HCl pH 8.0, 1 mM EDTA. The DNA was precipitated at -20^{o}C overnight by addition of 2.5 volumes of ethanol. The DNA was collected by centrifugation at 10,000 g for 30 minutes at 4^{o}C. (10,000 rpm Sorvall HB4), washed with 70% ethanol twice followed by centrifugation as before. This washed DNA was then dried <u>in vacuo</u> and reconstituted in sterile distilled water.

Small amounts of tRNA which interfered in the 5' labelling by kinasing were removed by chromatography on Sephacryl 300 (Pharmacia) in 10 mM Tris-HCl pH 7.5, 150 mM NaCl. The excluded peak was collected.

(26) <u>Alkaline plasmid preparation</u> (Birnboim and Doly 1979)

This method makes use of the stability of plasmid DNA to an alkaline treatment. An overnight culture of the plasmid containing bacteria (10 mL) was pelleted by centrifugation at 4,000 g for 5 minutes (6000 rpm Sorvall HB4) and the supernatant poured off. The pellet was resuspended in fresh 50 mM glucose, 10 mM EDTA, 2 mg/ml lysozyme and 25 mM Tris-HCl pH 8.0 (2 ml) and left on ice for 30 minutes. Then 4 mL of fresh 1% $NaDodSO_4$ (w/v) in 0.2 M NaOH was mixed in and after 5 minutes at 0^{o}C, 3 mL of 3 M $NaCH_3COO$ pH 4.8.

After a further 60 minutes at 0°C,this mixture was centrifuged at 4,000 g for 5 minutes (6,000 rpm Sorvall HB4) or at 6,000 g for 1 minute (12,000 rpm Eppendorf microfuge). The supernatant was carefully removed, and 2.5 volumes of ethanol added to it and the DNA was, precipitated at -20°C for 30 minutes and collected by centrifugation at 10,000 g for 15 mins or 6,000gfor 5 mins (10,000 rpm Sorvall HB4, 12,000 ppm Eppendorf). The DNA pellet was dissolved in 10 mM Tris-HCl pH 8.0 1 mM EDTA, and reprecipitated as above, 0.3 M NaCl and 2.5 volumes of ethanol. This was repeated again. After drying, the DNA was dissolved in water. Problems were encountered with nuclease which was the reason for the repeated precipitations, phenol extractions and or heating to 70°C for ≥ 60 seconds are alternatives which were used. This method for small quick preparations of plasmid DNA was largely superceded by the method below.

(27) Boiling Lysis plasmid preparation (Holmes and Quigley 1981)

A culture (5 mL) of the required bacteria containing plasmid was grown up overnight, centrifuged down at 4000 g for 5 minutes (6000 rpm Sorvall HB4) and the maximum amount of supernatant removed. To the pellet of bacteria was added the lytic mix (8% (w/v) sucrose, 5% (v/v) Triton X 100, 50 mM EDTA, 50 mM Tris-HCl, pH 8.0, (350 µl) and fresh 10 mg/mL lysozyme (25 µl)). The bacteria were resuspended and placed in a boiling water bath for 40 seconds and centrifuged immediately at 10,000 g for 10 minutes (10,000 rpm Sorvall HB4). The supernatant was removed and isopropanol (350 µL) added. This mixture was left at -18°C for 10 minutes to allow the

DNA to precipitate. The DNA was separated by centrifugation at 10,000 g for 5 minutes (10,000 rpm Sorvall HB4). The pellet was washed twice into 70% ethanol, vacuum dried, resuspended in sterile water (50 µL) and a sample (15 µL) run on a gel. This DNA does not appear to suffer the same degree of nuclease activity found for the alkaline method above.

(28) Transformation Protocol for Plasmid DNAs

The recipient E. coli was streaked out on an agar plate with appropriate medium (eg L Broth for HB101). A single colony was picked out and an overnight culture (50 mL) set up. This overnight culture (0.2 - 0.3 ml) was then diluted 1:100 and grown at 37°C to an absorbance at 600 nm of 0.6 (for HB101 this should take about 2 - 2.5 hrs). The cells (20 mL) were then pelleted by centrifugation at 4000 g for 5 minutes (6,000 rpm Sorvall HB4). The pelleted cells were resuspended in 50 mM $CaCl_2$ (10 mL) and incubated in ice water for 15 minutes. The cells were again pelleted as above, and taken up in 50 mM $CaCl_2$ (2 mL). These cells were used in transformations by mixing 200 µL of cells with 100 µL of DNA solution, (in 10 mM Tris-HCl pH 7.5, 10 mM $CaCl_2$ and 10 mM $MgCl_2$). The mixture of DNA and cells was incubated for 15 minutes at 0°C, then 5 minutes at 37°C, followed by the addition of 0.7 mL of medium to each tube which was then incubated for 30 minutes at 37°C. The cells were spread on to agar plates containing the medium supplemented with the selection antibiotic and incubated overnight at 37°C.

Ligation and Transformation of cDNA from 266BL poly A^+ RNA

The plasmid vector pAT153 was prepared by Hind III restriction endonuclease digestion followed by bacterial alkaline phosphatasing. Plasmid DNA (20 µg, prepared as above by cleared lysis) was digested with Hind III restriction endonuclease (25 units, Biolabs), the reaction was terminated by addition of $NaDodSO_4$ to 0.1% (w/v) and EDTA to 5 mM followed by extraction with 2 volumes of phenol ($CHCl_3$ (1/1, v/v), twice with 2 volumes of $CHCl_3$ and precipitation of the DNA with 2.5 volumes of ethanol at -20°C overnight. The restricted DNA was then treated with bacterial alkaline phosphatase (BRL) in a 50 µL incubation containing 50 mM Tris-HCl, 0.1% $NaDodSO_4$ (w/v) and 2.4 x 10^5u/ml of bacterial alkaline phosphatase, at 65°C for 30 minutes. The incubation was terminated by dilution and addition of $NaDodSO_4$ to 0.2%, EDTA to 5 mM and NaCl to 0.3 M, followed by two extractions with 2 volumes of phenol/$CHCl_3$ (1/1, v/v), two extractions with $CHCl_3$ and precipitation of the DNA with ethanol at -20°C overnight. After spinning out the DNA at 10,000 g for 20 minutes (10,000 rpm Sorvall HB4), and washing with ethanol, this Hind III restricted bacterial alkaline phosphatased pAT153 was redissolved to a concentration of 100 µg/ml. To test the prepared plasmid DNA, 100 ng was self ligated at 15°C for 8-12 hrs in 20 µL containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM ATP, 20 mM DTT and 10-20 u/mL T4 DNA ligase (BRL). In addition usually an identical reaction mixture containing additionally 100-75 u/ml T4 polynucleotide kinase was included to assess the ability of the plasmid to ligate after replacement of the 5' terminal phosphate. These reactions were used

to transform 200 µl of competent E. coli (see above) usually MRC8. If the plasmid did not show good background figures, i.e. a factor of ~1:1000 for dephosphorylated plasmid:rephosphorylated plasmid, and give a level of transformation close to that of supercoiled plasmid indicating that no degradation of the Hind III end had occurred, the procedure was repeated.

The ligation of the Hind III linkered, 'tailored' genes with plasmid was carried out at 15°C for 4-8 hr, as above, for testing plasmid. Typically, 100 ng of plasmid was ligated with 1-4 ng of the 'tailored' gene of average length ~1000 base pairs representing ~200 fold molar excess of plasmid. This mix was then used to transform E. coli, MRC8 as above. The colonies were grown on plates containing 100 µg/ml of carbenicillin (Sigma).

Colony Hybridisations

(30) Nitrocellulose filter hybridizations (Grunstein and Hogness 1975)

Colonies of the bacteria for screening were grown up overnight at 37°C on nitrocellulose filters (Millipore HA filters, 0.45 µM), which had been washed three times in boiling water (1 minute per wash), and overlaid on to an agar plate containing the appropriate medium. The cells were lifted with the nitrocellulose filter, placed on another agar plate with the appropriate medium supplemented with 170 µg/ml chloramphenicol and incubated overnight at 37°C to amplify the plasmids (Gergen et al., 1979). The nitrocellulose filters with amplified colonies were placed on a pad of 3 MM (Whatman) filters soaked in 0.5 M NaOH, to lyse the colonies and incubated for 7 minutes. These lysed colonies on the nitrocellulose filters were then transferred to a fresh pad of 3 MM filters, soaked in 1 M Tris-HCl pH 7.5 for 2 minutes, followed by transfer to a pad of 3 MM filters soaked in 0.5 M Tris-HCl pH 7.5 and 1.5 M NaCl and left for 7 minutes. The filter was then placed on a vacuum manifold and the colonies sucked down by a vacuum pump, washed with ~100 mL of ethanol, air dried and baked at 80°C - 85°C for 2 hr.

These filters with immobilized DNA could be stored at this stage. For hybridization, the filters were first prehybridized in order to block any free binding sites on the nitrocellulose filter. This prehybridization is carried out in 2 X SSC (0.2 M NaCl, 0.015 M sodium citrate), 10 x DH (Denhardts, 1966; 0.2% (w/v) of each of ficoll (MW 400,000), polyvinylpyrollidone (MW 360,000) and bovine serum albumin), 50 mM Na_2HPO_4/NaH_2PO_4 pH 7.0, 400 µg/ml, single stranded

sonicated salmon sperm (S5) DNA and 100 µg/ml poly A (PL Biochemicals) at 60°C for 2-4 hr. The addition of poly A was only made for the use of hybridization probes containing poly A or poly T sequences to prevent nonspecific hybridization to these sequences, it was omitted for hybridization using oligodeoxynucleotides.

The hybridization conditions were as above with the addition of the probe with incubation overnight at 60°C after denaturation by heating to 100°C for 5 minutes in water. Initial hybridizations were done under mineral oil after saturating the filter in hybridization mix, but later sealed plastic bags were used for both prehybridizations and hybridizations with the prehybridization medium being squeezed out of the bag prior to addition of hybridization mix. The hybridized filters under mineral oil were washed with $CHCl_3$ to remove the oil, followed by washing in 2 X SSC four times at room temperature for 5 minutes each and two times in 0.1 X SSC, 15 minutes each, at 55°C.

The hybridization of filters with oligodeoxynucleotide probes was carried out as above, but at various temperatures in the range 26-35°C, and washed in 2 x SSC at room temperature and temperatures up to 35°C. The amount of oligonucleotide used was 0.1 - 2.0 pmole per filter in 3 mL of hybridization mix, after 5' labelling with kinase as described earlier. The filters were exposed at -70°C with intensifying screens (Ilford) and X ray film (Kodak Xomat).

(31) Whatman No 540 filter hybridizations (Gergen et al., 1979, Wallace et al., 1981).

Colonies for screening were grown up on agar plates with the appropriate medium. After overnight growth, colonies were lifted onto Whatman 540 filters by laying dry filters down on to the agar

plate and smoothing down if necessary. The filter and colonies were then incubated for a few hours (1-2 hr). The filters were then lifted off with the attached colonies and laid with colonies down on to an agar plate containing in addition to the normal media, 170 µg/mL chloramphenicol, and incubated overnight. The colonies were lifted off with the filter and lysed by washing the filters twice for 5 minutes in each of the following solutions, successively, 0.5 M NaOH, 0.5 M Tris-HCl pH 7.5, 2 x SSC pH 7. The filters with the now immobilized DNA were washed briefly in 95% (v/v) ethanol and air dried.

These filters could be stored or used directly for hybridization. For hybridization the filters were prehybridized in 100 µg/ml S5 DNA, 6 x SSC, 0.5% NP40, 5 mM EDTA and 50 mM $Na_2PO_4/NaHPO_4$ pH 7.0 at 60^{o}- 55^{o}C for 1-2 hr. The hybridization conditions for the 11-14 base oligodeoxynucleotides were 0.25 pmoles/mL of the oligomer, 200 µg/mL S5 DNA, 6 x SSC, 0.5% NP40, 5 mM EDTA and 50 mM $Na_2PO_4/NaHPO_4$ pH 7.0 at 35^{o}C overnight (Wallace et al., 1981). The conditions for the 22 base oligonucleotide were as for the oligomers above but with 3 x SSC at 55^{o}C with the probe at 1 - 0.3 x 10^6 cpm/mL. The washing conditions were 6 x SSC at 0^{o}C for 4 washes of 10 minutes each, followed by increased stringency of washing in 6 x SSC up to the hybridization temperatures for a short time (10-20 min).

(32) Labelling Deoxyribonucleic acid by nick-translation

(Maniatis et al., 1975 and Rigby et al., 1977)

This method uses the ability of E. coli DNA polymerase I to catalyze a "nick-translation" reaction, coupling the sequential addition of nucleotide residues to the 3'-hydroxyl terminus of a nick with the elimination of the nucleotide units from the nick's 5'-phosphoryl terminus. The nicks are introduced by the use of DNAase I, bovine pancreatic (DPFF, Worthington Biochemical Corp.).

The radioactive nucleotides to be incorporated were dried down in an Eppendorf tube in a vacuum, 100 µCi (400 Ci/mmol, Amersham) each of (α^{32}P) dTTP and (α^{32}P) dCTP. To this tube was added 2 µl of 65 µM dATP and dGTP, 3.3 µl of 10 x "Nic mix"(0.5 M Tris-HCl pH 7.5, 0.05 M $MgCl_2$ and 0.1 M 2-mercaptoethanol), 2.6 µl of DNAase I (2 ng/ml) 4 units of DNA polymerase I (Boehringer, complete enzyme) and 0.5 µg of DNA to be labelled. The solution was made up to 33 µl with H_2O and incubated at 15°C for 1-3 hours. The DNAase I was freshly diluted in water from a 2 mg/ml stock, 1 in 10^6. The reaction was stopped by addition of 100 µl of "stop mix" (10 mM Tris-HCl pH 7.5, 0.5% (w/v) $NaDodSO_4$ and 100 µg/mL of S5 DNA), followed by extraction with 2 volumes of water saturated phenol/$CHCl_3$ (1/1, v/v). The interface was re-extracted with 100 µl of "stop mix", the aqueous phases pooled and run on a Sephadex superfine G50 (Pharmacia, 0.7 cm x 18 cm) column, equilibrated in 3 x SSC. The excluded peak was collected, and DNA was precipitated by the addition of 2.5 volumes of ethanol, followed by incubation at -70°C for 1-2 hours, and collected by centrifugation at 10,000 g for

15 minutes (10,000 rpm Sorvall HB4). This DNA was ready for use in hybridizations, the specific activity at $\sim 10^8$ cpm/µg of nick translated DNA.

(33) Labelling and Construction of the 22 base oligodeoxynucleotide

(Doel et al., 1980)

The construction of the 22 oligodeoxynucleotide probe was done by 5' end labelling of the oligomer B and its ligation to the oligomer A using oligomer C as the template, see fig 4-9.

The label to be used, 100 µCi of (γ^{32}P) ATP (>5000 Ci/mmol, Amersham) was dried down in vacuo and taken up in 10 µl containing 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM 2 mercaptoethanol, 6µM oligonucleotide B, and 200-500 u/mL of the T4 polynucleotide kinase (PL Biochemicals). This mix was incubated at 37°C for 30 minutes followed by incubation at 65°C for 5 minutes to inactivate the kinase. This mix was diluted to 50 µl, made 2 µM with respect to oligonucleotide A, 1.6 µM to oligonucleotide C (these also being heated at 65°C for 5 minutes prior to addition), and 1.2 µM to oligonucleotide B in 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 20 mM DTT, 1 mM ATP and 25 u/mL T4 DNA ligase (BRL) and incubated at 25°C for 2-4 hours. This reaction was terminated by addition of an equal volume of "urea mix" (10 M urea, 5 mM Tris-borate pH 8.3, 0.1 mM EDTA, 20% sucrose, 0.1% (w/v) xylene cyanol FF and 0.1% (w/v) bromophenol blue and heating at 65°C for 5 minutes and then loaded on to a 15% acrylamide, 0.5% bisacrylamide, 7 M urea TBE, polyacrylamide gel. The ligated 22 oligonucleotide probe was visualized by autoradiography and the band cut out and

eluted as described.

Hybridization to filter bound fractionated nucleic acid

(34) (Southern 1979, Thomas 1980 and Wallace et al., 1981)

The nucleic acids (RNA and DNA) transferred to nitrocellulose as described earlier (Southern 1979 and Thomas 1980) were available for probing with radioactively labelled probes, nick translated double stranded DNA or 5' labelled oligodeoxynucleotides, generated as described. In this study nick-translated DNA was used to probe RNA on filters essentially as described (Thomas 1980) and a 22 base oligodeoxynucleotide (Chapter 5) was used to probe DNA on filters (Wallace et al., 1981).

The filters with the immobilized poly A^+ RNA were prehybridised in 50% (v/v) deionized formamide, 5 x SSC, 50 mM Na_2HPO_4/NaH_2PO_4 pH 7.0, 250 µg/mL S5 DNA, 1 x DH, 0.1% (w/v) $NaDodSO_4$ and 10 µg/mL poly A at 42^oC for 2-4 hours. This mix was removed and replaced by a mix constructed as for the prehybridization, but containing the nucleic acid probe denatured at 100^oC for 5 minutes, with the addition of 0.2 volumes of 50% (w/v) dextran sulphate (Sigma). The medium in the bag was then mixed to get an even spread of probe and incubated overnight at 42^oC. After hybridization the filters were washed in 2 x SSC, 0.1% $NaDodSO_4$ at room temperature four times 5 minutes each, followed by 2 washes of 15 minutes with 0.1 x SSC, 0.1% $NaDodSO_4$ at 50^oC.

The filters with immobilized DNA fragments were prehybridized in 3 x SSC, 50 mM Na_2HPO_4/NaH_2PO_4 pH 7.0, 0.1% (w/v) $NaDodSO_4$,

10 x DH and 80 µg/mL S5 DNA at 55°C for 2-3 hours. This was followed by removal of the prehybridization mix and replacement with the same mix containing the 22 base oligonucleotide probe at 1-0.3 x 10^6 cpm/mL. The filter was hybridized overnight at 55°C. After hybridization filters were washed with 4 changes of 6 x SSC at 0°C.for 30 minutes followed by a 5 minute wash at 55°C for 5 minutes.

Both RNA and DNA filters after hybridization were autoradiographed at -70°C using intensifying screens (Ilford) and Xray film (Kodak, X-Omat or Fuji RX).

The RNA filters were reused by washing the filters in 2.5 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 0.025% sodium pyrophosphate and 0.05 x DH at 65°C for 2 hours. The filters were then prehybridized and hybridized as before.

Sequencing DNA (Maxam and Gilbert, 1977, Air et al., 1976).

(35) The sequencing was performed as described by Maxam and Gilbert (1980), where good details of the reaction steps are given. The base cleavage reactions used in this study were C+T, C, G and A+C. The DNA for sequencing in this study was plasmid DNA purified by CsCl gradient followed by Sephacryl S300 (Pharmacia) column purification, the excluded peak being collected. The DNA was cut with restriction endonucleases to produce 5' single stranded termini, which were more easily labelled by the following procedure.

Plasmid DNA (10 µg) was digested with an appropriate restriction endonuclease followed by phenol extraction. The digested plasmid was then treated with bacterial alkaline phosphatase as described for Hind III digested pAT 153 (see earlier molecular cloning of poly A^+ RNA, ligation and transformation).

The digested DNA with dephosphorylated 5' ends was then kinased. The label, 100 µCi of ($\gamma^{32}P$) ATP (>5000 Ci/mmol, Amersham) was dried down in vacuo, taken up in 25 µL of 50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 2 mM DTT, 0.5 mM spermidine, 0.1 mM EDTA and 400 u/mL T4 polynucleotide kinase (PL Biochemicals), followed by incubation at 37°C for 30 minutes. The reaction was terminated by addition of 100 µL of 2.5 M ammonium acetate, 1 µL tRNA (0.5 mg/mL) and 375 µL of ethanol followed by incubation at -70°C for 5 minutes and the DNA was spun down at 9,960 g for 5 min (12,000 rpm Microfuge). If the labelled DNA fragments were to be fractionated prior to the second restriction digest, the DNA pellet was dried in vacuo and taken up in

TBE gel loading buffer and subjected to gel electrophoresis (see DNA electrophoresis). If the DNA was to be subjected to re-restriction prior to fractionation, the pellet was redissolved in 125 µL of 0.3 M, CH_3COONa and reprecipitated with 3 volumes of ethanol as above. The DNA pellet was washed with 95% (v/v) ethanol and dried in vacuo. This DNA was then subjected to some test digestions with various restriction enzymes, in order to determine the best enzyme to generate suitable fragments labelled at one 5' end. The DNA fragments labelled at both 5' ends, (either before or after gel purification) were redigested with a second restriction endonuclease and purified by gel electrophoresis, as described earlier, but with addition of 40 µg of carrier DNA (sonicated Calf thymus DNA) prior to precipitation.

The washed, dried DNA pellet was taken up in 22 µL of H_2O and measured out into Eppendorf tubes for the four chemical cleavage reactions, 4.5 µL for G and C, 5.5 µl for A+C and C+T. The A+C reaction was carried out by addition of 20 µL of 1.5 M NaOH, 1 mM EDTA with incubation at 90°C for 5-30 minutes (50-100 bases for 10 minutes, >100 bases for 5 minutes), followed by neutralisation with 30 µL of 1N HCl, 200 µL of 0.2 M Tris-HCl pH 7.5 and 2 µL tRNA 10 mg/mL. The G reaction was carried out by addition of 200 µL "DMS buffer" (50 mM sodium cacodylate pH 8.0, 10 mM $MgCl_2$, 0.1 mM EDTA) cooling to 0°C, addition of 1 µL of dimethyl sulphate (10 M) and incubation at 24°C for 5-30 minutes (50-100 bases for 10 min, >100 bases for 5 min). The reaction was terminated by addition of 50 µL of "DMS stop" (1 M Tris-acetate pH 7.5, 1 M 2-mercaptoethanol, 1.5 M sodium acetate, 50 mM $Mg(CH_3COO)_2$, 1 mM EDTA and 0.4 mg/mL tRNA). The T+C reaction was carried out by addition of 26 µL of H_2O, cooling to 0°C, addition of 30 µL of 95-100% hydrazine (Kodak) and incubation at 25°C

for 10-40 minutes (50-100 bases for 15 min, >100 bases for 10 min). The C reaction was the same as for T+C but with addition of 26 µL of 5 M NaCl in place of 26 µL H_2O and times of 15-60 minutes at 24°C (50-100 bases for 15 min, >100 bases for 25 min). The T+C and C reactions were terminated with the addition of 200 µL "hydrazine stop" (0.3 M $NaCH_3COO$, 0.1 mM EDTA, 0.1 mg/mL tRNA).

Nucleic acid was precipitated from reaction mixtures by the addition of 0.8 mL of ethanol, incubation at -70°C for 5 minutes, and separated by centrifugation in a microfuge 9,950 g for 5 minutes (12,000 rpm Eppendorf). The DNA pellets were taken up in 0.25 mL of 0.3 M CH_3COONa pH 7.5, by warming at 37°C for 2 minutes and reprecipitated with the addition of 0.8 mL of ethanol, as above. The DNA pellet was dried in vacuo, the DNA then taken up in 100 µL of 0.9 M piperidine (redistilled) and heated at 90°C for 30 minutes to achieve cleavage of the modified DNA strands. This reaction was terminated by the addition of 10 µL of 2 M NaCl, 300 µL of ethanol and incubated at -20°C overnight to precipitate the DNA fragments. The DNA fragments were collected by centrifugation as above and washed with 0.5 mL ethanol and dried in vacuo for 15-30 minutes. These DNA pellets were taken up in loading buffer (95% deionized formamide, 20 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol FF and 25 mM Tris-borate pH 8.3), using 2-3 µL per loading. The gels used to resolve the DNA fragments were 0.6 mm thick TBE/ polyacrylamide gels (see DNA electrophoresis) in 7 M urea, either 8% acrylamide, 0.25% bisacrylamide gels, or for longer fragments >200 bases 6% acrylamide, 0.2% bisacrylamide gels. The

approximate position of certain nucleotide lengths on the gels was indicated by the position of the dyes (A. Porter personal communication: 6% gels with xylene cyanol (XC) at 105 bases and bromophenol blue (BPB) at 29 bases, 8% gels XC 65 bases; BPB 18 bases; 12% gels XC 50 bases, BPB 14 bases; 15% gels XC 40 bases, BPB 11 bases).

The gels after electrophoresis were wrapped in a plastic disposable bag and autoradiographed at -20°C with intensifying screens (Ilford) unless weak signals were present when they were autoradiographed at -70°C.

Immunoelectron Microscopy

(36) Immunoelectron microscopy was carried out by a modification of the method described previously (Bernadac and Lazdunski 1981). E. coli HB101 containing plasmids pSC213 and pWT211 were grown to a culture A_{600} of 0.2, induced with 3-β-indole acrylic acid and harvested at A_{600} of 3.0. The bacterial pellets were fixed by resuspension in a mixture of 2% formaldehyde and 0.2% glutaraldehyde in PBS for 1 hr at $0^{o}C$. The fixed E. coli were pelleted and pellets were cut under PBS into 1 mm cubes and dehydrated by immersion through a graded series of ethanols (50, 70, 80, 90 and 100% v/v). Specimens were embedded in Spurr's resin (Spurr 1969) using 1,2-epoxypropane as an intermediary. Polymerised blocks containing the specimens were sectioned on a Reichert OMU_3 Ultramicrotome using a diamond knife. Sections were mounted on copper mesh grids coated with collodion. Grids were incubated successively with goat IgG (0.5 mg/ml), either normal (non-immune) or ε chain specific in PBS, 0.1% NP40 for 1 hr at $20^{o}C$. Grids were washed with PBS, 0.1% NP40 and incubated with ferritin-labelled rabbit anti-goat IgG (Miles) at 1/100 dilution in PBS, 0.1% NP40 for 30 min at $20^{o}C$. The IgG's were initially preabsorbed with a solid phase overnight at $4^{o}C$ prepared from a sonicate of E. coli HB101 reacted with cyanogen bromide activated Sepharose 4B (Pharmacia). The IgG's were tested for activity after this by an Ouchterlony precipitation (Feinberg 1957). Excess IgG on the grids was removed by washing with PBS, 0.1% NP40. Grids with bound IgG were then stabilised by cross-linking in 0.25%

(w/v) glutaraldehyde in PBS for 1 hr at 20°C, followed by staining with magnesium uranyl acetate (0.5% in water) for 30 min at 20°C.

CHAPTER 3

The Isolation of Messenger RNA and translation in vitro

Poly A^+ RNA isolated from two human cell lines, 266BL a myeloma cell line which produces IgE (ε and λ chains) (Nilsson et al., 1970) and Bristol (Bri 7) a lymphoblastoid cell line which produces IgG (γ and κ chain) Molgaard et al., 1981). As immunoglobulin translating mRNA is associated with the rough endoplasmic reticulum (Pryme et al., 1973), initial attempts were made to prepare RNA from microsomes to enable some degree of purification for the Ig ε and λ coding mRNA from the 266BL myeloma cell line (3)*. Results obtained with the human lymphoblastoid cell line RPMI 1788 (Molgaard et al., 1981), indicated that active mRNA could be obtained by this method. Initial results with frozen cell pellets of the 266BL cell line gave low yields of poly A^+ RNA (2 µg/gm of cell pellet) with no detectable translational activity in the mRNA dependent rabbit reticulocyte lysate. This prompted the search for alternative procedures, with the problem of inhibiting or inactivating RNAase activity being the primary consideration. The use of rapid protein denaturation methods appeared to be the best way of rapid RNAase inactivation. The procedures using protein denaturants such as guanidine hydrochloride or phenol, in the presence of RNAase inhibitors such as heparin, iodoacetate and detergent were effective, but most effective was the use of guanidinium thiocyanate, which gave intact RNA from the pancreas a rich source of RNAase (Chirgwin et al., 1979). This latter method was chosen, its potential stemming from the cation and the anion being potent chaotropic agents. The

* Numbers in brackets refer to numbers of relevant methods.

method was tested using a protocol recommended by Dr. P. Butterworth, which makes use of 2-mercaptoethanol, a potent RNAase inhibitor (4), (Chirgwin et al., 1979). The initial results with RNA isolated from Bri7 demonstrated good yields of 2.8 mg of RNA and 68 µg of poly A^+ RNA/gm of Bri7 frozen cells. These results indicated the usefulness of the method, and it was applied to the cell line 266BL which gave yields of 1 mg of RNA and 3 µg of poly A^+ RNA/gm of frozen cells. These yields were markedly lower than those for Bri7, but translation in the rabbit reticulocyte lysate demonstrated adequate activity (Figure 3-1), which was not found for the microsome preparation (2,3). The possibility that the oligo dT cellulose column used in preparation of the poly A^+ RNA was not functioning efficiently was tested by translation of poly A- RNA see Figure 3-1, and by rerunning of poly A- RNA over oligo dT cellulose. This showed a markedly differing translational profile for poly A- RNA, and also little retention of further poly A^+ RNA, only what might be expected due to small amounts of nonspecific binding. Immunoprecipitation of the poly A- RNA translation products, indicated a significant proportion of Ig ε translational activity was associated with poly A^+ RNA.(9).

The possibility that the frozen cell pellets of 266BL provided by G. D. Searle (J. Birch) had degraded the RNA prior to extraction was considered and fresh cells were grown, (1). These fresh cells were processed without delay and also the subsequent handling of the RNA was reduced in order to deminish the possibilities of RNA degradation. Dr. J. M. B. Warwick (Personal communication) compared poly A^+ RNA prepared by guanidium thiocyanate and by phenol/pH 9 extraction

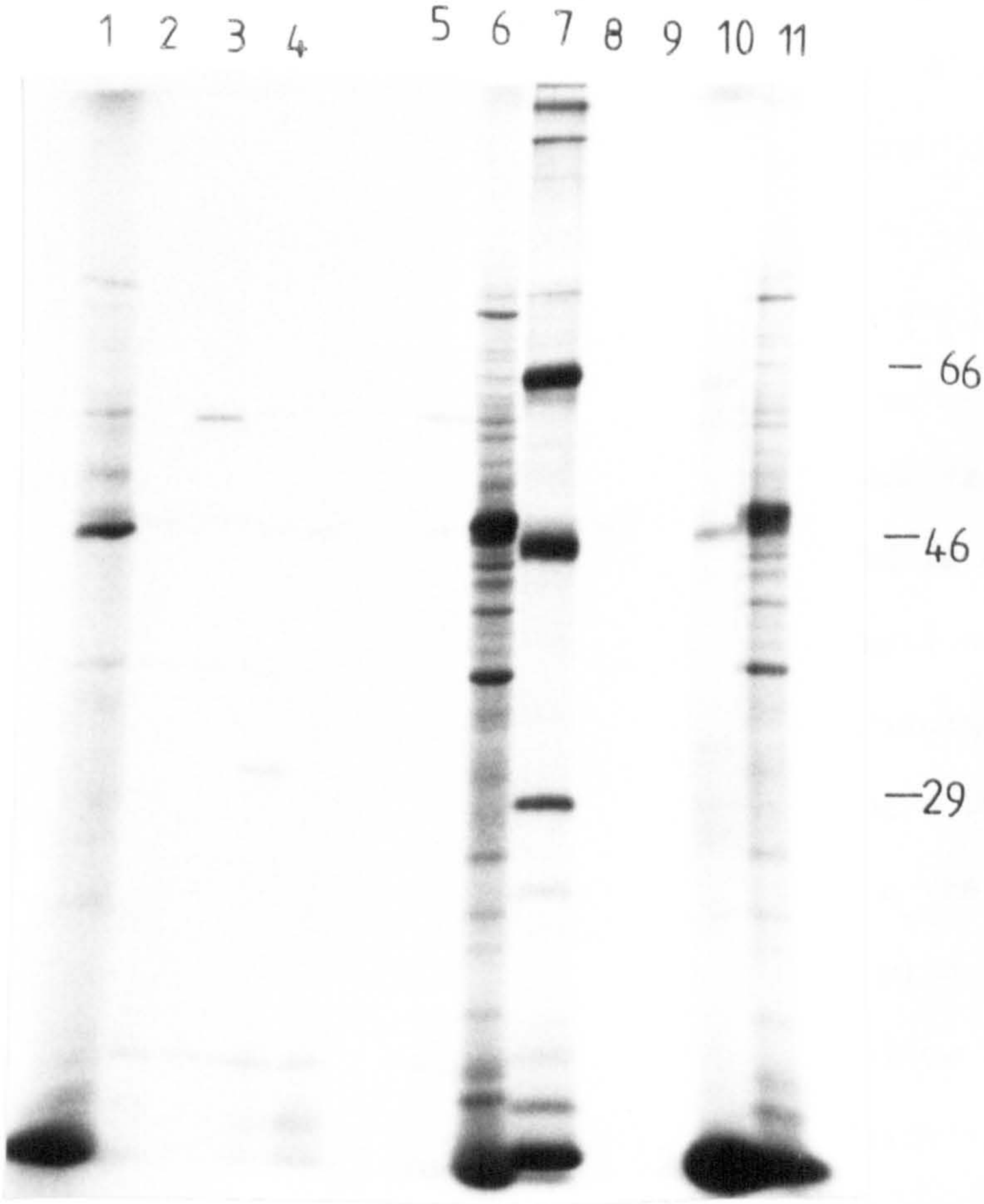


Figure 3.1 In vitro protein translation of 266 BL poly A+ RNA. Fluorograph of [^{35}S] methionine-labeled polypeptide translation products from 266 BL poly A+ and poly A-RNA, isolated using guanidinium thiocyanate.

(1) Poly A-RNA translation products; (6, 11) Poly A+ RNA translation products; (2-4) sequential immunoprecipitations with (2) anti ovalbumin (3) anti Ig E Fc (4) anti λ light chain; immunoprecipitations with (5) anti μ (8) anti ovalbumin; (9) immunoprecipitation with anti ε; (10) rabbit globin mRNA translation products; (7) [^{14}C] labeled molecular weight standards. Indicated molecular weights in daltons x 10^{-3} shorter exposure.

(4A,B) from the human lymphoblastoid cell line RPMI 1788 and found that in the mRNA dependent rabbit reticulocyte lysate translation system, the poly A^+ RNA prepared by the phenol/pH 9 extraction method was markedly superior in activity, this also appeared to hold for a tissue cultured human fibroblast cell line. Accordingly we used the phenol/pH 9 extraction method on the fresh cells. The yield of first pass oligo dT cellulose purified poly A^+ RNA was 25 µg/mg of total RNA, in comparison to expected yields of 2.5% poly A^+ RNA from total RNA at this stage (Faust et al., 1979) (5). This yield is markedly higher, even allowing for an expected two fold contamination with ribosomal RNA (from first pass oligo dT cellulose purification), than that found in the guanidinium thiocyanate method of 3 µg of poly A^+ RNA/mg RNA. The translation of both poly A^+ RNAs in the mRNA dependent rabbit reticulocyte lysate (Figure 3-2) was, with a two fold greater amount of phenol/pH 9 prepared poly A^+RNA, to allow for a degree of contamination from rRNA, this demonstrates the superiority of this phenol/pH 9 prepared poly A^+ RNA. It is possible that the difference was either due to, or exaggerated by, the use of fresh cells. All subsequent poly A^+ RNA preparations were made from fresh cells using phenol/pH 9 and provided suitable RNA.

Immunoprecipitation with anti serum specific for Ig ε heavy chains (Miles) or Ig E ND Fc (Pharmacia) demonstrated the presence of a translation product of 61,000 ± 1,000 daltons (figure 3-1) (7,9,10). This is close to an estimated molecular weight for Ig ε heavy chain

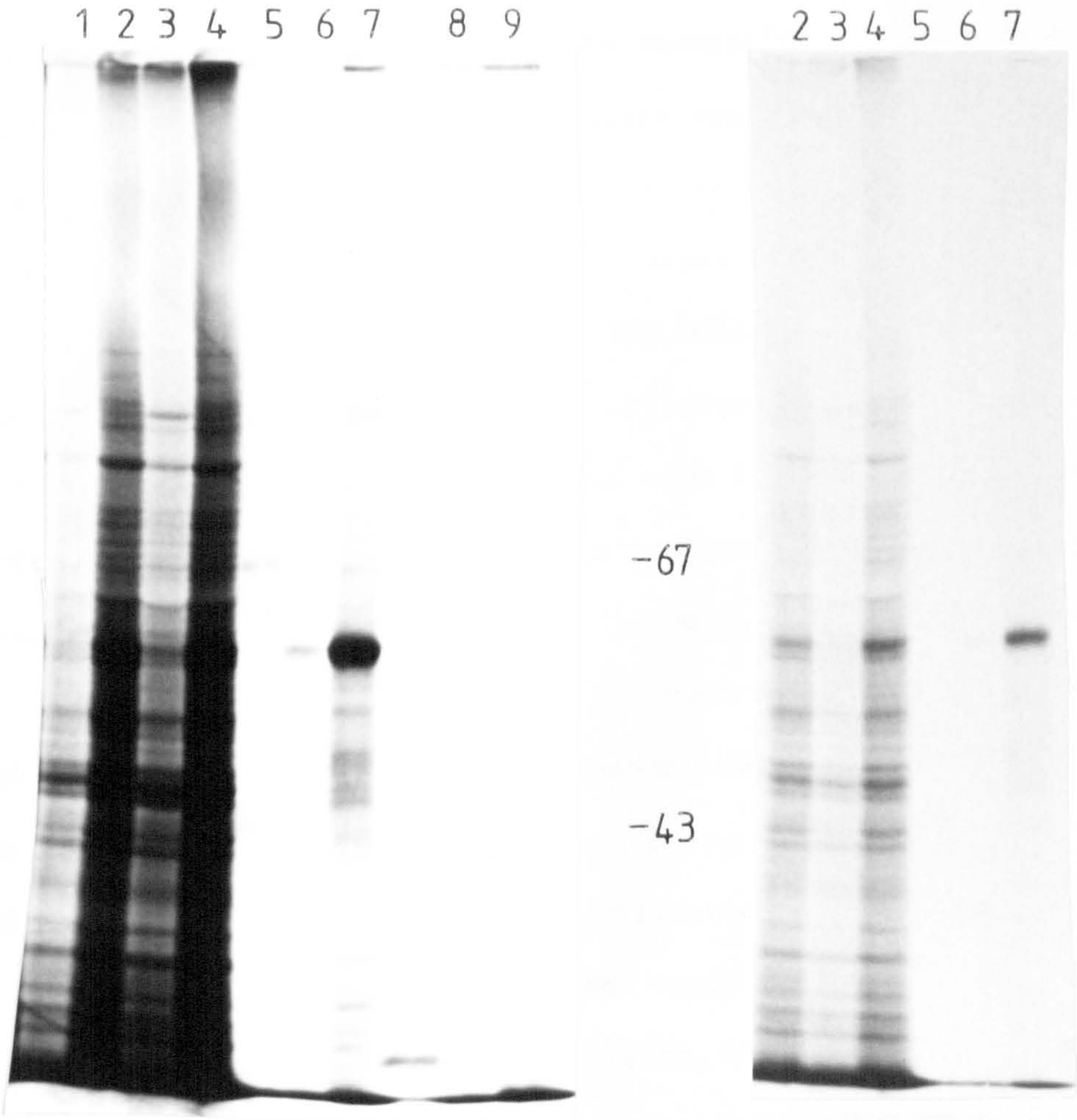


Figure 3.2 In vitro protein translation of 266 BL poly A+ RNA from two isolation methods.

Fluorograph of [^{35}S] methionine-labeled polypeptide translation products from guanidinium thiocyanate prepared poly A+ RNA (1, 3, 6, 8); and from phenol/pH9 prepared RNA (2, 4, 7, 9). Total translation products (3, 4); immunoprecipitates of total translation products; anti Ig E Fc (6, 7), anti Ig μ heavy chain (8, 9). Total translation products after anti Ig E Fc immunoprecipitation (1, 2). [^{14}C] labeled molecular weight markers (5). Indicated molecular weights in daltons x 10^{-3}.

determined from the available protein sequence data (Dorrington and Bennich 1978) of 61,000 daltons. The translation product from the mRNA dependent rabbit reticulocyte lysate would be expected to contain a pre-peptide as a signal for secretion. The size for the pre-peptide would be expected to be 19 amino acids (Methyssens and Rabbitts 1980), resulting in an expected molecular weight of 63,000 daltons. The molecular weight does not significantly differ from the value of 61,000 obtained, which is also in agreement with the values found for the rat pre-Ig ε heavy chain (Zajdel-Blair et al.,1981; Hellman et al., 1982; Faust and Moore, 1981).

In order to reduce the number of clones needing to be screened in the subsequent cloning of complementary DNA (cDNA) copies of the mRNA, further purification of the Ig ε heavy chain coding mRNA from poly A^+ RNA was attempted. Purification was attempted by the use of sucrose gradients, two gradient types being used (6). Initial attempts were with linear 5-30% gradients, the first result is shown in Figure 3-3. The translation and immunoprecipitation of gradient fractions is shown in Figure 3-4. The smearing of Ig ε precipitable activity over a large area of the gradient was unexpected. The sucrose gradient run was repeated as above,giving the result as shown in Figures 3-5 and 3-6. This also showed some degree of smearing of the Ig ε heavy chain translational activity. Subsequent attempts were with an isokinetic gradient 15-32% sucrose, without any NaCl (6). It was hoped these more denaturing conditions would lead to less spreading of the Ig ε heavy chain translational activity, due to a reduction of the levels of secondary

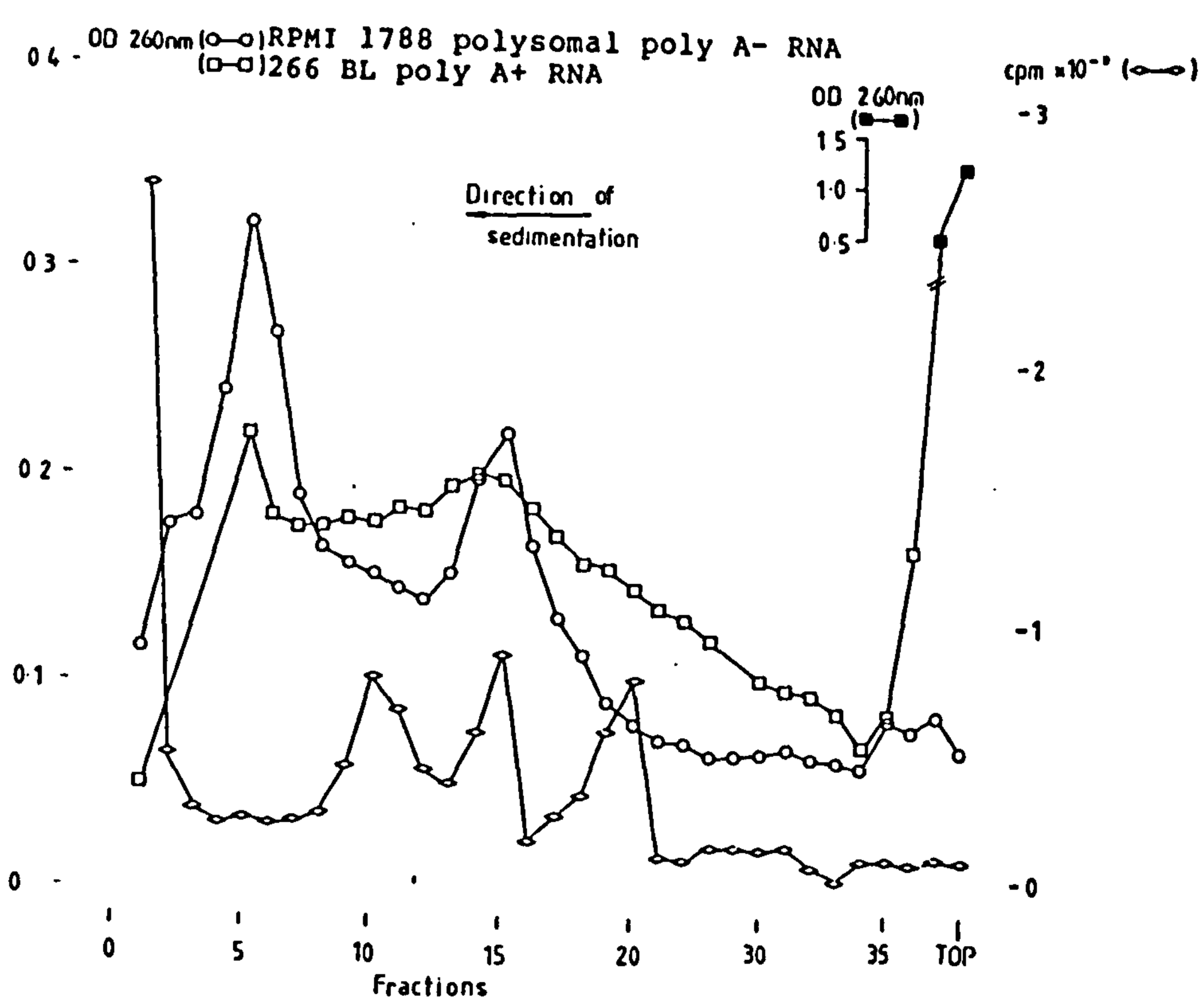


Figure 3.3 Sucrose gradient profile of 60 µg of RPMI 1788 polysomal poly A- RNA as 28S and 18S markers, open circles with [^{14}C] labeled E. coli RNA 4S, 16S and 23S markers, diamonds; and the profile of 80 µg of 266 BL poly A+ RNA in a parallel gradient open and filled squares.

< Direction of sedimentation.

16 15 14 13 12 11 E M 10 9 8 7 6 5

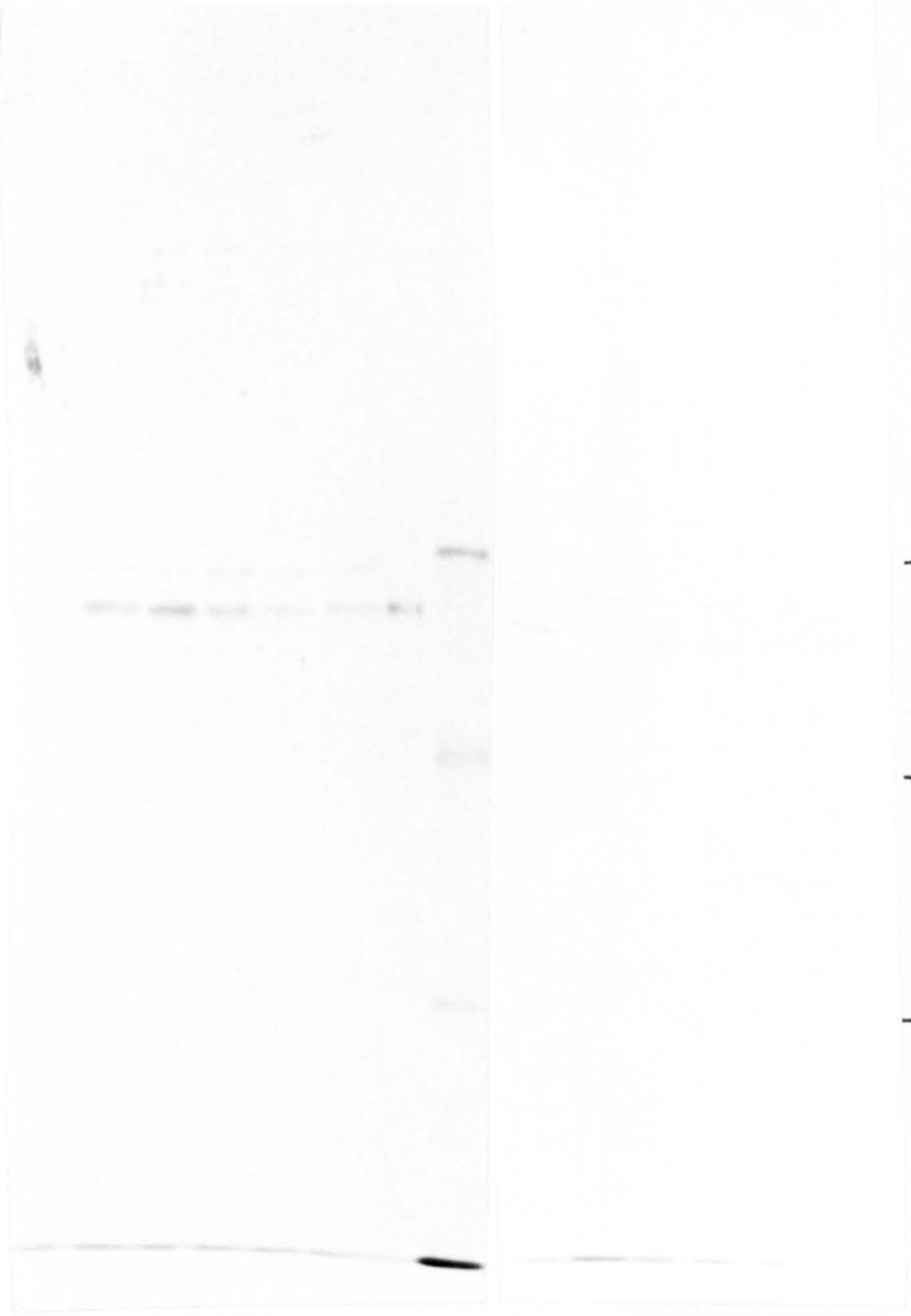


Figure 3.4 In vitro protein translation of fractions from 5 - 30% linear sucrose gradient (Figure 3.3). Fluorograph of [^{35}S] methionine-labeled polypeptide immunoprecipitated for Ig E Fc, synthesised from poly A+ RNA purified by sucrose density gradient centrifugation. Fractions from gradient 5-16. [^{14}C] labeled molecular weight standards as in materials and methods (M). Immunoprecipitate for Ig E Fc, from translation of total poly A+ RNA prior to sucrose gradient (E).

Indicated molecular weights in daltons x 10^{-3}.

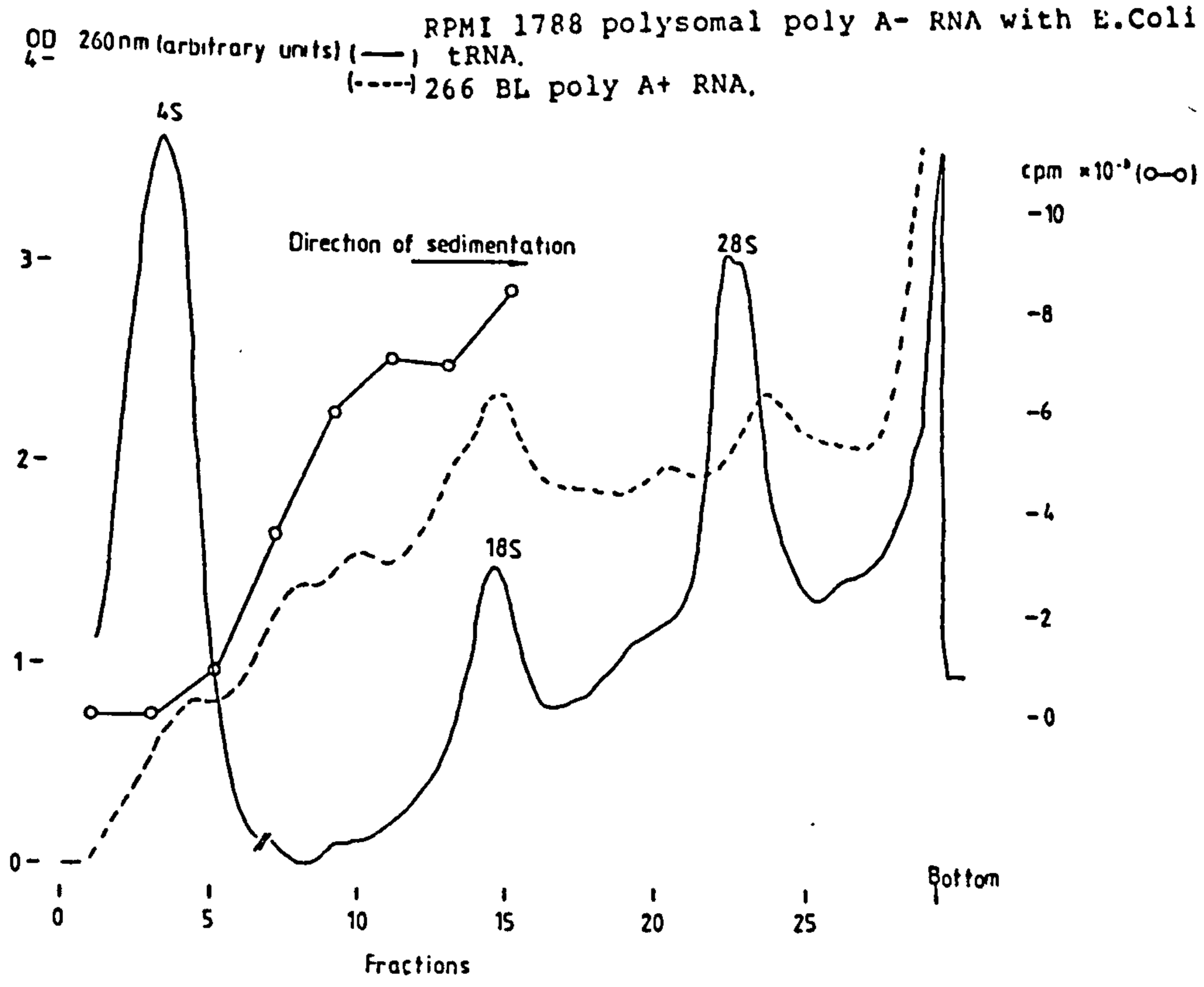


Figure 3.5 Sucrose gradient profile of 60 µg of RPMI 1788 polysomal poly A- RNA, with 20 µg of E. coli tRNA, as 28S, 18S and 4S markers, and 100 µg of 266 BL poly A+ RNA. Open circles, the translational activity of certain fractions is plotted as [^{35}S] methionine incorporation (cpm x 10^{-3}) into TCA insoluble material in the rabbit reticulocyte in vitro translation system.

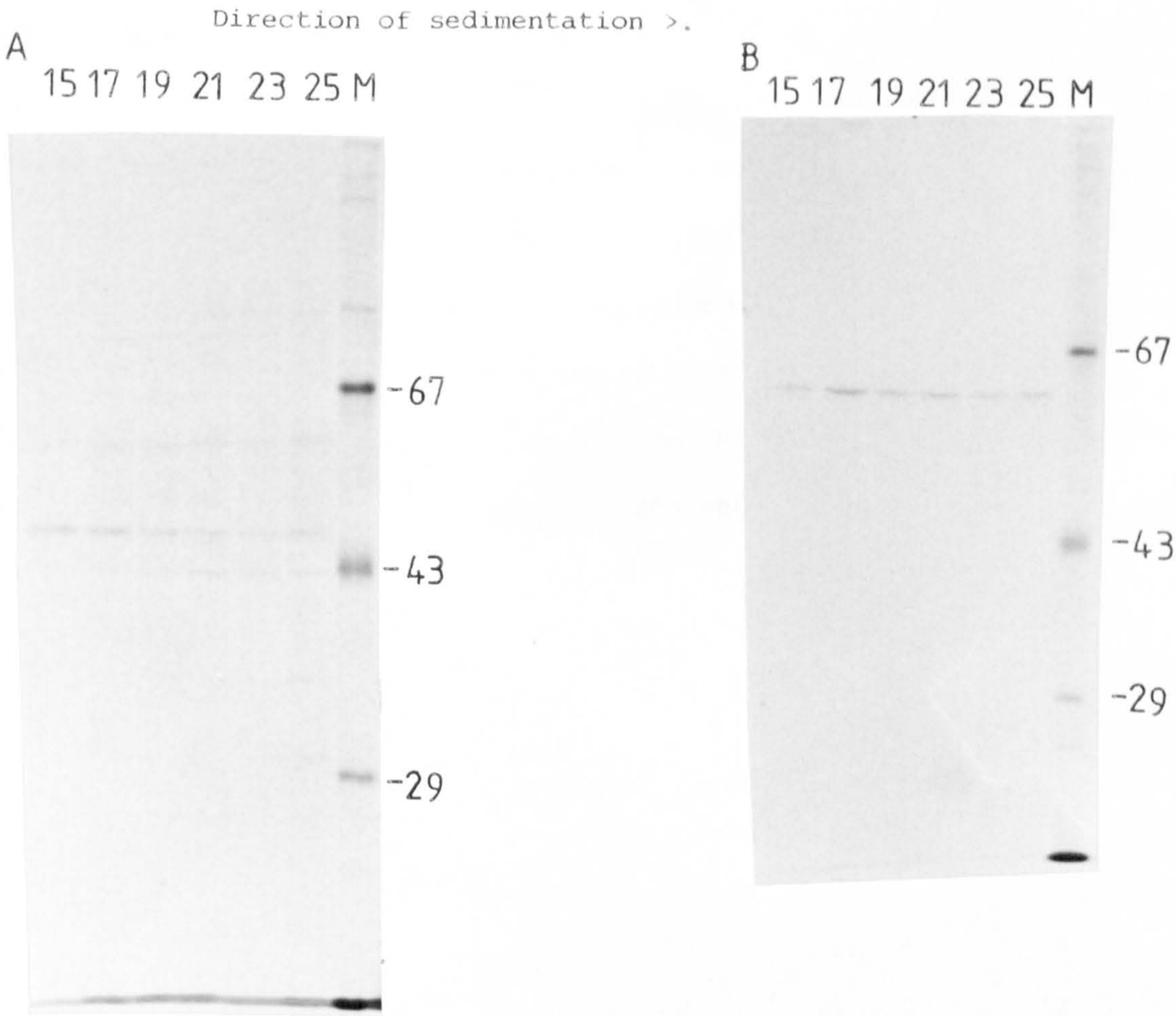


Figure 3.6 In vitro translation of sucrose gradient purified RNA corresponding to the sucrose gradient shown in Figure (3-5). Fluorograph of [^{35}S] methionine-labeled polypeptides synthesised from sucrose gradient purified poly A+ RNA in a rabbit reticulocyte lysate. 1 µl of total translation products: A fractions 25, 23, 21, 19, 17, 15; immunoprecipitations of 10 µl of translation products, for Ig E Fc B fractions 25, 23, 21, 19, 17, 15; [^{14}C] labeled molecular weight markers AM, BM. Molecular weights in daltons x 10^{-3}.

structure possible for the RNA. The gradient run was made with BRL labelled RNA Standards (^{14}C), 16S, 23S; (^{3}H) 4S, 18S, 28S, in a parallel gradient, unfortunately these were degraded. Fractions from the sucrose gradient were translated (Figure 3-7) and immunoprecipitated for Ig ε heavy chain translation products (Figure 3-8). This gradient demonstrated a sharper peak of Ig ε heavy chain translational activity. Material prepared on this gradient was used subsequently for some priming experiments and cloning of the Ig ε mRNA.

< Direction of sedimentation.

11 12 13 14 M 15 16 17 18

Figure 3.7 In vitro translation of RNA from sucrose gradient.

Fluorograph of [^{35}S] methionine labeled polypeptides synthesised from sucrose gradient purified poly A+ RNAs in a rabbit reticulocyte lysate. 1 µl of total translation products of fractions 11 - 18 from sucrose gradient, (28 fractions in total); Molecular weight markers (M) labeled with [^{14}C]. Molecular weights in daltons x 10^{-3}.

< Direction of sedimentation.

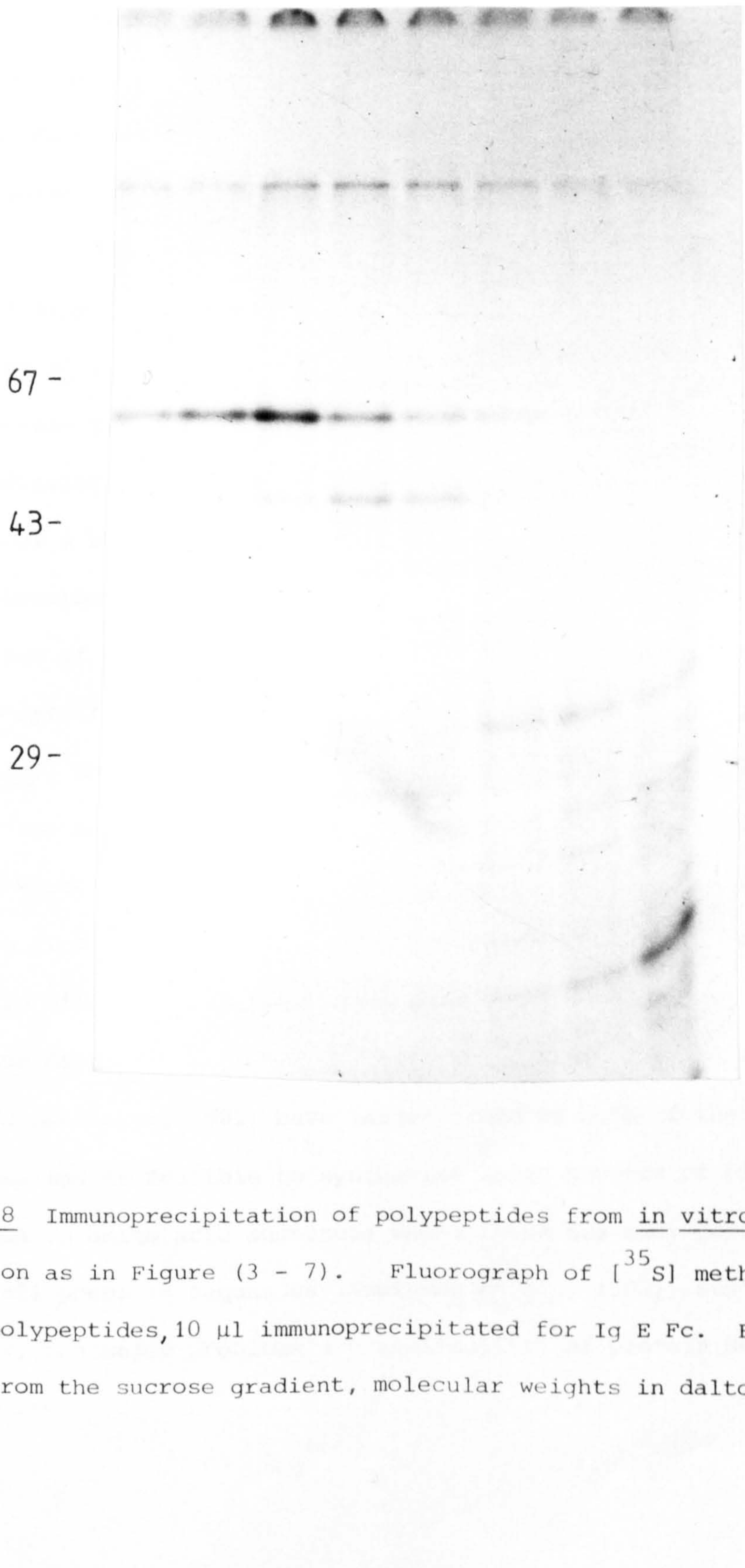


Figure 3.8 Immunoprecipitation of polypeptides from in vitro translation as in Figure (3 - 7). Fluorograph of [^{35}S] methionine labeled polypeptides, 10 µl immunoprecipitated for Ig E Fc. Fractions 11 - 18 from the sucrose gradient, molecular weights in daltons x 10^{-3}.

CHAPTER 4

Primed Synthesis of cDNA

Deoxyoligonucleotide primers for AMV reverse transcription on poly A^+ RNA has been widely used for a long time for the synthesis of single stranded complementary DNA (cDNA), the deoxyoligonucleotide being dT_{12-15}, using poly A tails found on most eukaryotic mRNAs (Ross et al., 1972). This use of oligonucleotide primers has enabled the cloning of many mRNAs (Rabbitts, 1976; Gough et al., 1980; Mac Reynolds et al., 1977). The second strand of the cDNA is constructed by AMV reverse transcriptase or DNA polymerase I, with the self priming activity of single stranded cDNA, typically through the formation of a hair pin structure. The hair pin is removed by SI nuclease treatment prior to insertion into cloning vectors.

The use of deoxyoligonucleotides has developed over recent years with more specific oligonucleotide sequences, as priming templates (Chan et al., 1979; Noyes et al., 1979, Smith et al., 1979). Initially the main problem was the lack of suitable amino acid sequences which could be encoded by few complementary oligonucleotide primers (e.g. <4) and so reduce the problems of cost and effort involved in oligonucleotide synthesis. The recent advances in solid phase deoxyoligonucleotide synthesis (Miyoshi et al., 1980, Tanaka and Letsinger, 1982) have largely removed both of these problems making it feasible to synthesise large numbers of oligo-nucleotides to amino acid sequences where there are many possibilities, covering all possible sequences (Sumikawa et al., 1982; Suggs et al., 1981), now, the major problems are availability of protein sequence

data and of sufficient mRNA.

With the Ig ε heavy chain most of the protein sequence had been determined for the ND myeloma (Dorrington and Bennich 1978). This data enabled the selection of a suitable amino acid sequence for the design of a deoxyoligonucleotide primer. The sequence chosen Trp-Gln-Glu-Lys for the 11 nucleotide sequence, enabled all possibilities to be covered in 4 sequences as below.

Protein	NH_2		Trp	Gln	Glu	Lys	COOH
mRNA		5'	UGG	CAA	GAA	AA A	3'
					G	G G	
Primers	(P_1)	3'	ACC	GTC	CTC	TT	5'
	(P_2)		ACC	GTT	CTC	TT	
	(P_3)		ACC	GTC	CTT	TT	
	(P_4)		ACC	GTT	CTT	TT	

These oligonucleotides were synthesised by the Chemistry Dept., of G. D. Searle as described (Houghton et al., 1980a). The chosen amino acid sequence is located near to the COOH termini of the Ig ε chain starting at amino acid 516 (Dorrington and Bennich, 1978). The primer directed synthesis of cDNA on 266BL poly A^+ RNA should result in a specifically primed cDNA of ∿1,800 bases long. Statistically generation of primed cDNA of this length should be a rarer event than shorter transcripts, as the number of sequences in the poly A^+ RNA population capable of generating such a transcript will be small. This will be valuable as the primed cDNA products generated by 11 oligonucleotide primers are not unique (Chan et al., 1979; Noyes et al., 1979; Houghton et al., 1980a).

The initial results with primer P1 to P4 are shown in Figure 4-1, (17) this reaction did not demonstrate the expected transcript. There was the possibility that the obtained transcripts were premature termination products as had been described for globin and Chorion mRNA templates (Efstratiadis et al., 1975). In consequence, primer directed transcripts labelled 1 to 3 on figure 4-1 were extracted from the gel slices for sequencing directly by Maxam and Gilbert, (1977) Methods (35). The base modification reactions A>C,G, T+C were for 30 minutes and C for 40 minutes. The final cleaved fragments were run on a 0.6 mm thick 8% sequencing gel (Bromophenol Blue run to 2/3 of gel length) followed by exposure at -70°C for 3 weeks. The sequences obtained did not relate to the expected sequence for an Ig ε mRNA, cDNA. This prompted a scale up of priming reactions in order to obtain sufficient labelled longer cDNAs for sequencing. Selected reactions, i.e. those most promising for generation of long transcripts from Figure 4-1, were scaled up 4 fold, these were those corresponding to tracks 2 and 8 (Fig 4-1). The band corresponding to "a" in Figure 4-1, track 2, was extracted for sequencing but with only 2,050 Cherenkov c.p.m. extracted in this cDNA little if any sequence was expected. The small amount of sequence data discernible after a 4 week exposure at -70°C indicated it was not Ig ε cDNA. These results were not unexpected in the absence of a significant cDNA transcript of the expected length.

The possibility that conditions were not favourable for the priming reaction was considered. The level of primer used may not have been sufficient although in an excess when calculated, i.e. a

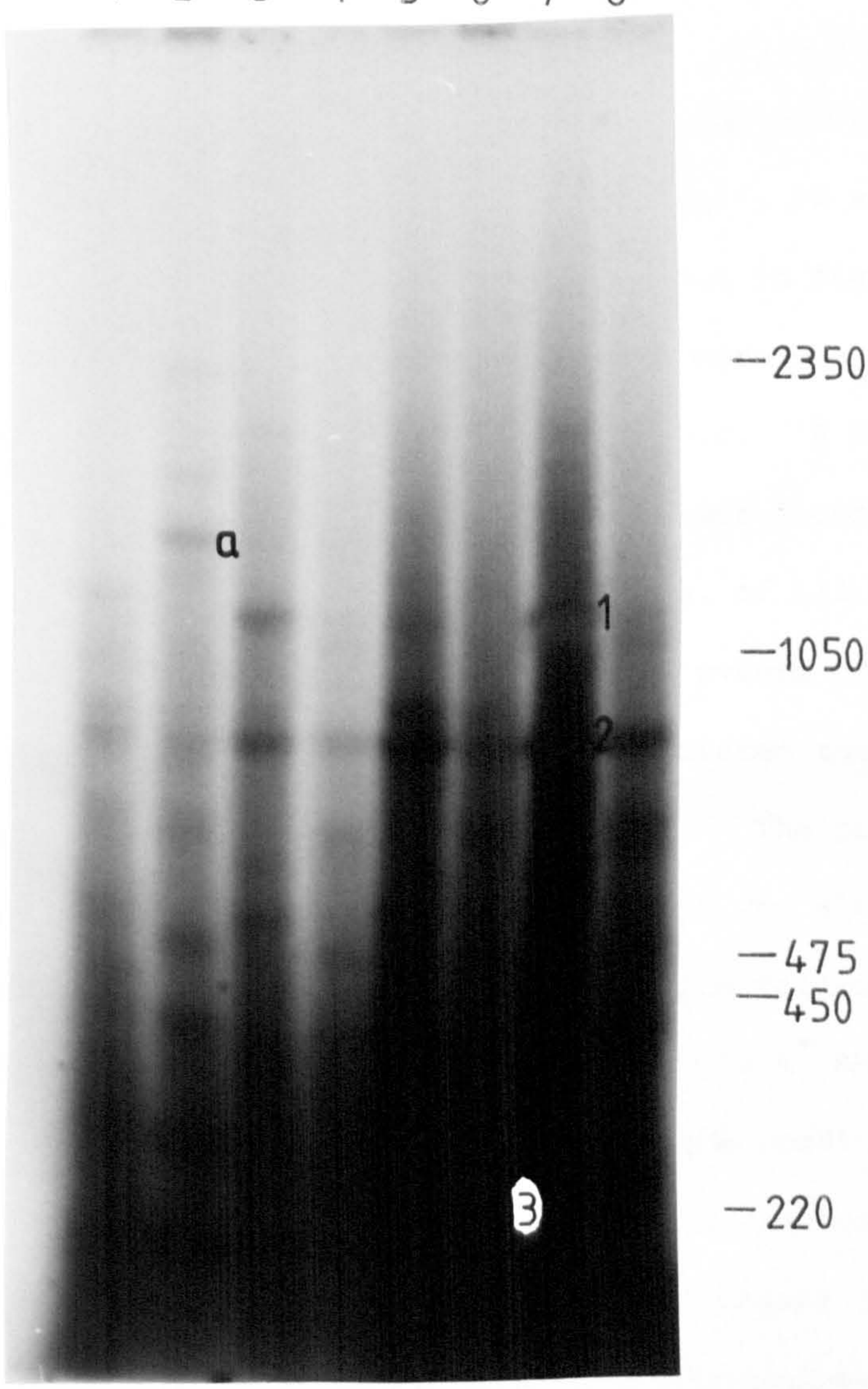


Figure 4.1 Alkaline gel electrophoresis of primed cDNA transcripts from poly A+ RNA using synthetic oligodeoxynucleotides. Tracks 1 - 4 poly A+ RNA prepared using the guanidinium thiocyanate procedure, tracks 5 - 8 poly A+ prepared using the phenol/pH9 procedure. Transcripts from oligodeoxynucleotide P1, tracks 1 and 5; P2, tracks 2 and 6; P3, tracks 3 and 7; P4, tracks 4 and 8. Sizes indicated in bases.

base molar excess of 1.6 fold and a complementary molar excess of 6.8×10^3 fold (from allowing for frequency of 11 nucleotide sequences complementary (1 in 4.2×10^6 bases), to an average size for the mRNA of 1,000 bases). To evaluate 'excess', P2 was incubated at differing primer to RNA levels as shown in figure 4-2. The results demonstrated that addition of 10 fold more RNA produced significantly raised incorporation into primed bands. A 2 fold increase in primer did not sigificantly affect primer incorporation into primed bands, except into shorter transcripts, of ill defined length, probably relating to non-specific priming events due to high concentration of primer. This experiment demonstrated the limiting effect of the RNA species in the priming reaction. The potential affect of hybridizing temperature on priming events was also studied, by brief heating of poly A^+ RNA at 90^oC for 1 minute followed by hybridization for 1 hour at 40^oC with primer and poly A^+ RNA and then normal AMV reverse transcription Figure 4-3. These conditions were chosen because it might have been important to reduce secondary structure in the poly A^+ RNA in order to allow the primer access to potentially hidden sites. Little effect was however observed, except a reduction in the level of transcripts. However, in subsequent work Agarwal et al., (1981) were able to demonstrate some effects of temperature on the secondary structure.

These results indicated the general application of the methods used, particularly since they had proved successful (Houghton et al., 1980a; Houghton et al., 1980b). The determination of the oligonucleotide sequences was the next check to try. The possibility

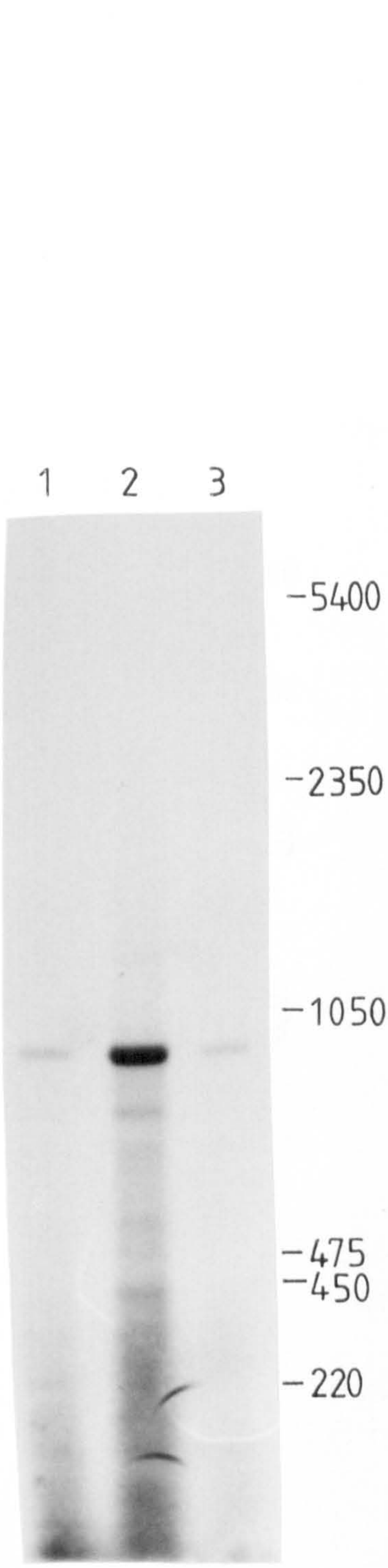


Figure 4.2 Alkaline gel electrophoresis of primed cDNA transcripts from poly A+ RNA using synthetic oligodeoxynucleotide P2. Track (3) 'Normal' RNA and P2 levels, track (1) 2 fold increase in P2 levels, track (2) 10 fold increase in RNA levels. Sizes indicated in bases.

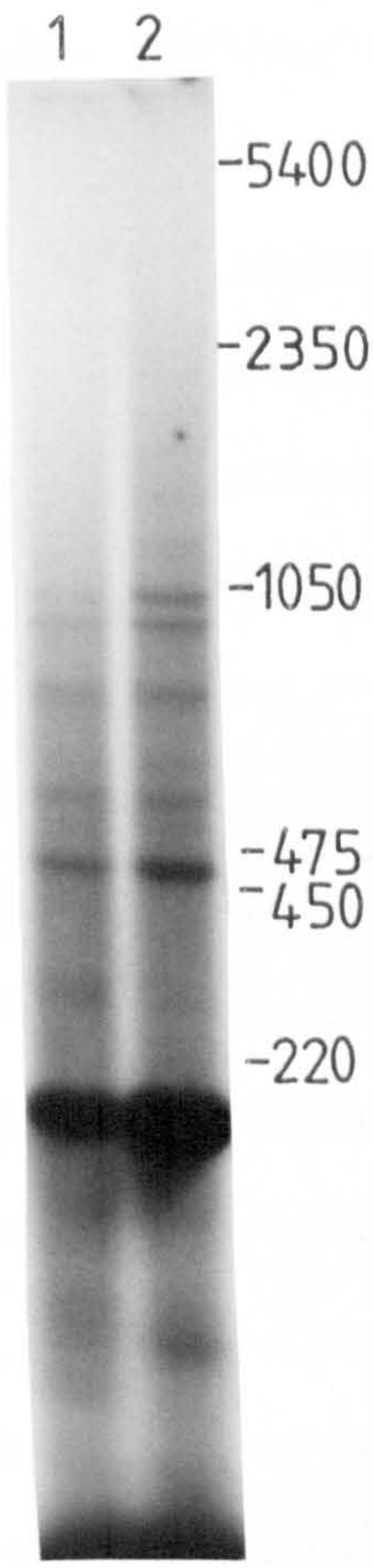


Figure 4.3 Alkaline gel electrophoresis of primed cDNA transcripts from poly A+ RNA using synthetic oligodeoxynucleotide P3.

Track (2) 'Normal' conditions for hybridisation.

Track (1) raised temperature for hybridisation (see text).

Sizes indicated in bases.

that these were incorrect seemed unlikely in view of the success of the Chemistry Dept., in synthesis of oligonucleotides. The primers were subjected to sequence analysis by the wandering spot technique, the results (Figure 4-4) showing the correct sequences, only the last 5' nucleotide not being determined (16). This technique is more exacting than Maxam and Gilbert sequencing since low levels of incorrect bases at each step are detected due to the cumulative effect on the spot pattern, e.g. for a 12 nucleotide sequence with 10% error at each nucleotide might not be detected by Maxam and Gilbert with only 31% of the correct sequence. The 3' end used to initiate cDNA synthesis was demonstrated to be correct. The use of 11 deoxyoligo-nucleotide primers containing mismatched base pairs, e.g. an 11 nucleotide primer with a base pair, mismatch at position 3 from the 5' end (Houghton et al., 1980a), has worked, indicating that even with the 5' nucleotide incorrect they will prime. The publication of the gene sequence (Max et al., 1982; Flanagan and Rabbitts, 1982) later provided the reasons for failure, incorrect protein sequence.

Under the direction of the Chemistry Dept., at G. D. Searle, (Dr. M. A. W. Eaton) 2 new 11 oligonucleotide primers were synthesised for a new region of the human Ig ε starting at amino acid 468 (Dorrington and Bennich, 1978). Of the 4 possibilities the 2 synthe-sised were:

amino acids	NH_2	Asn	Phe	Met	Pro	COOH
mRNA	5'	AAC	UUC	AUG	CCN	
		U	U			
Primer (P_5)	3'	TTA	AAG	TAC	GG	
(P_6)		TTG	AAG	TAC	GG	

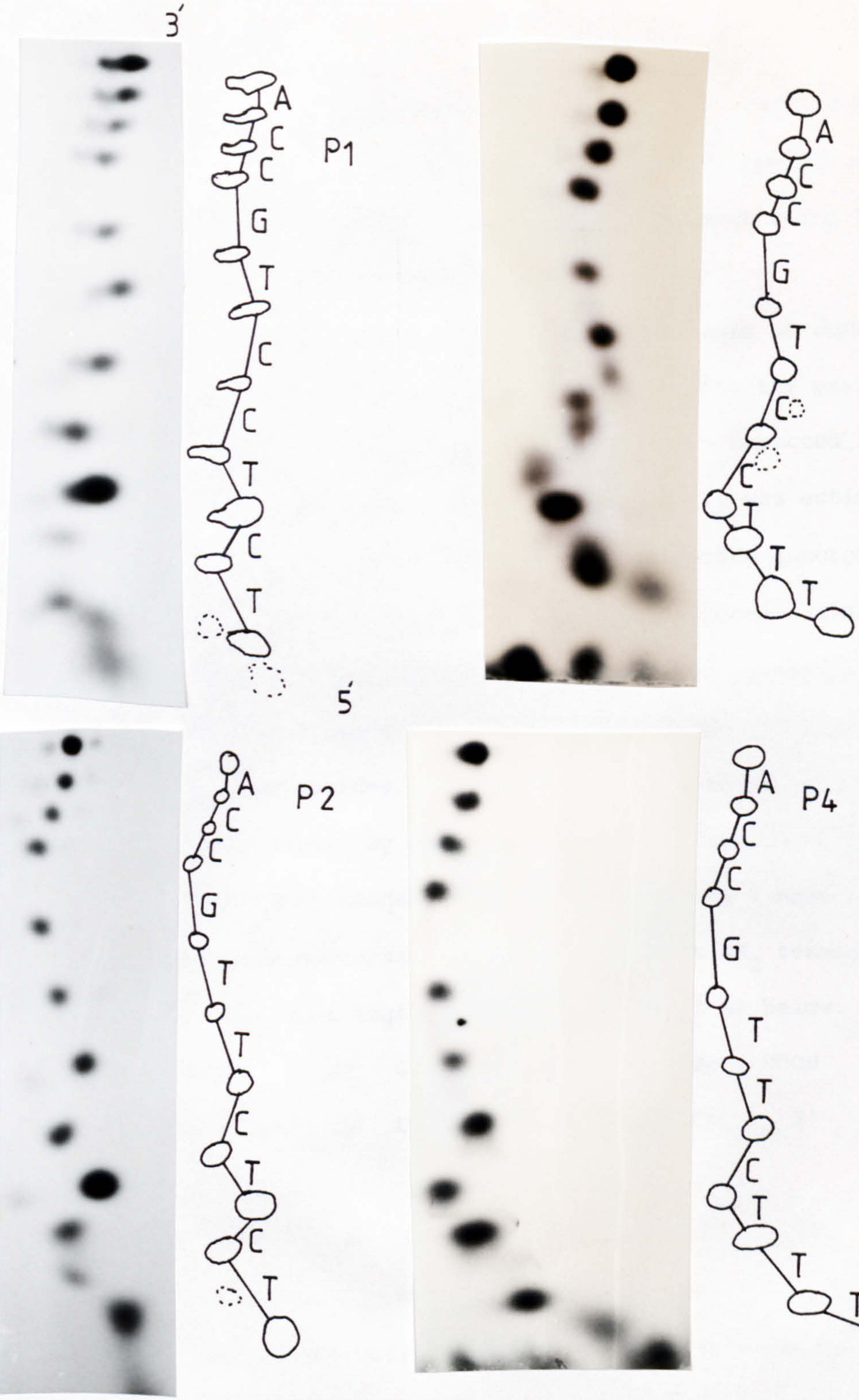


Figure 4.4 Two dimensional fingerprint of a partial snake venom phosphodiesterase of the synthetic oligodeoxynucleotides P1 to 4, with a diagrammatic representation of the sequences.

The preferred codon for Phe, TTC was used to reduce the possible complementary 11 nucleotide sequences to 2. This preferred codon shows strong usage in immunoglobulin genes sequences being 31 to 10 in favour of this triplet (Grantham et al., 1981).

These two primers were used to direct synthesis of cDNA on total poly A^+ RNA, see Figure 4-5. Primer P6 with the preferred anti codon for Asn, produced a primed band of the expected length, 1700 bases. This priming activity was low and it was estimated that large amounts of RNA would be needed to provide incorporation for sequence analysis. Because of this, other possibilities were initially explored. During the synthesis of the primers P5 and P6 an intermediate of 7 bases was stored to allow for the construction of longer oligonucleotides. Longer oligonucleotides were constructed by the Chemistry Dept.. by synthesis of two 7 oligonucleotide sequences followed by condensation to the previous 7 base intermediate. This extended oligonucleotide covers to the next NH_2 terminal amino acid, Gln No. 467 (Dorrington and Bennich, 1978) as below:

amino acids	NH_2	Gln	Asn	Phe	Met	Pro	COOH
mRNA	5'	CAA	AAC	UUC	AUG	CCN	3'
		G	U	U			
Primers (P_7)	3'	GTT	TTG	AAG	TAC	GG	5'
(P_8)		GTC	TTG	AAG	TAC	GG	

These two primers were tested for priming activity on poly A^+ RNA, with a raised preincubation temperature from 25^oC to 28^oC. This was to reduce mRNA secondary structure and allow the formation of a possibly more stable mRNA to 14 deoxyoligonucleotide hybrid. The

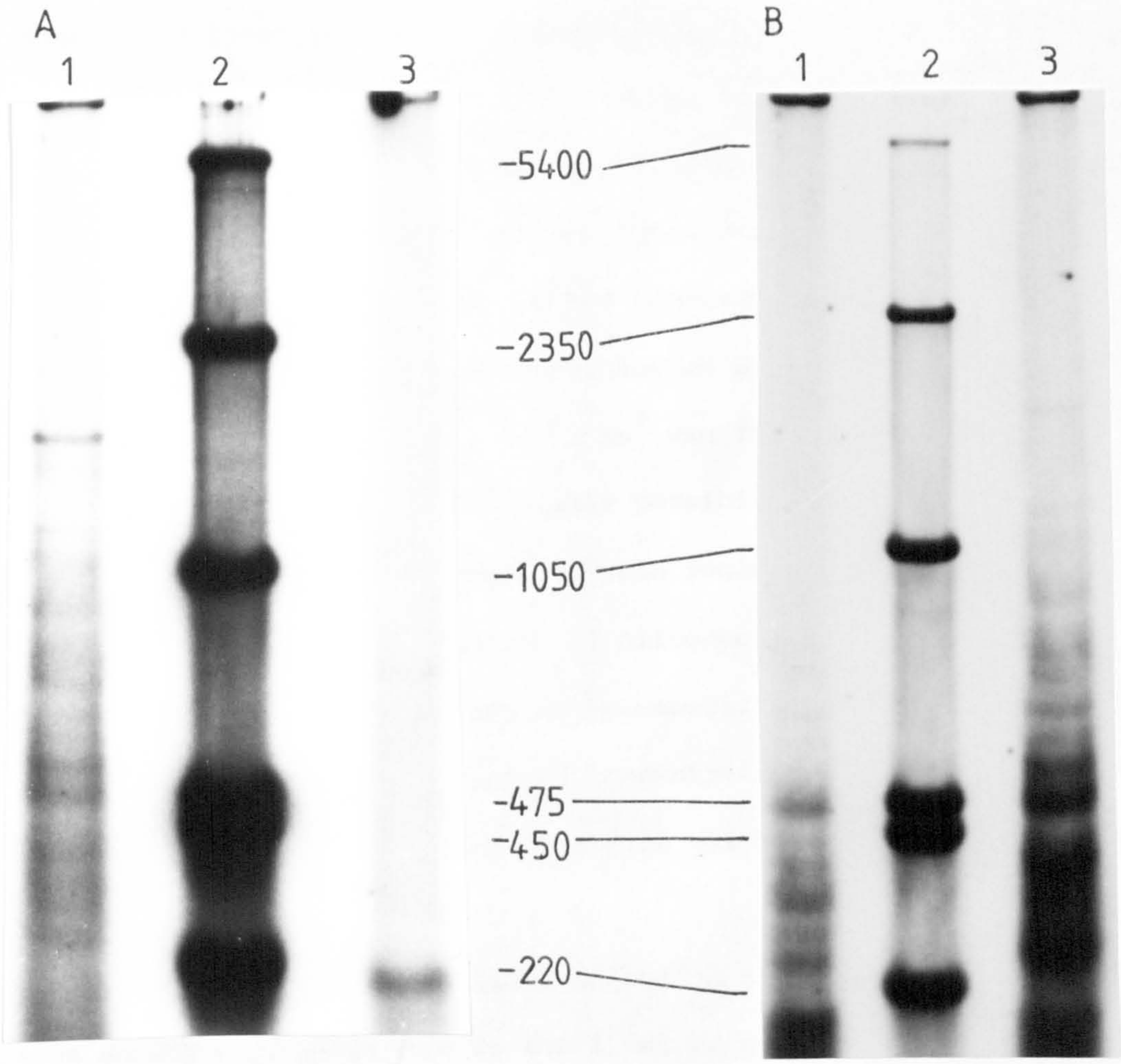


Figure 4.5 Transcription products of 266 BL mRNA. [^{32}P]labeled cDNA products were analyzed by alkaline agarose gel electrophoresis

(A) Transcripts generated by oligodeoxynucleotide primers P6,

3' T-T-G-A-A-G-T-A-C-G-G 5' (lane 1) and P5, 3' T-T-A-A-A-G-T-A-C-G-G 5'

(lane 3). Lane 2 shows the Hind III digest of phage PM2 DNA containing the fragments of lengths (in bases) as indicated.

(B) Transcripts generated by oligodeoxynucleotides P7,

3' G-T-T-T-T-G-A-A-G-T-A-C-G-G 5' (lane 1) and P8,

3' G-T-C-T-T-G-A-A-G-T-A-C-G-G 5' (lane 3). Lane 2 shows the Hind III digest as in Figure 4-5 (A) above.

results are shown in Figure 4-5, showing the 1,700 base band but exhibiting reduced intensity with stronger priming of the shorter cDNAs. The result was unexpected, the possibility that the pre-hybridization of the mRNA to primer temperature had been raised too high was considered unlikely, as the increase in temperature of dissociation between an 11 oligonucleotide and a 14 oligonucleotide of identical G, C content in 0.9 M Na^+ was from 33.2°C to 40.6°C (Wallace et al., 1979). The likely possibilities were that this new sequence competed unfavourably with regions of secondary structure not encountered by the 11 oligonucleotide, or that its new sequences were hybridizing to non-specific RNAs resulting in reduction of the effective primer concentration, or that this next amino acid used for extension of the 11 oligonucleotide sequence is incorrect.

The estimation of the level of the Ig ε mRNA from the incorporation of labelled primer P6 to the 1,700 base transcript was carried out. The assumptions made were an average size of mRNA of 1,000 bases, a 40% incorporation of 5,000 Ci/mmol (^{32}P) PO_4 into the primer, a 50% level of mRNA from the poly A^+ RNA, and a 50% priming efficiency. These factors allowed an estimate from one experiment of Ig ε in RNA at 0.024% of total mRNA. (640 cpm Cerenkov/μg of poly A^+ RNA). If this were true it would explain the low level of incorporation in priming reactions.

The possibility of using the 14 oligonucleotide primer as a probe in screening for bacterial colonies containing cloned Ig ε heavy chain RNA sequences (Wallace et al., 1979; Wallace et al., 1981;

Suggs et al., 1981) was tested. Initially cloned material was screened, but no colonies gave sufficiently consistent results to be considered as Ig ε clones by hybridization (Chapter 5).

Screening colonies using the primed cDNA transcript has been used (Chan et al., 1979), in our case the 5' labelled cDNA transcript proved to be labelled insufficiently to be used to screen a large number of colonies. The possibility of generating an internally labelled cDNA transcript was tested using 10 μCi of (α^{32}P) dATP, with 0.1 mM dATP, and 0.6 μM primer in a typical priming reaction. The result is shown in Figure 4-6, demonstrating the potential of this approach. The reaction was scaled up using 1 mCi of (α^{32}P) dATP to provide sufficient labelled probe for screening, but this probe failed to give suitable results although hybridizing to certain colonies, (Chapter 5).

It was important to ascertain if the 1,700 base primed cDNA was an Ig ε mRNA transcript, in view of the negative results obtained for screening, before undertaking further work. The results obtained by reduction of the potential secondary structure of poly A^+ RNA and primer together, by heating followed by slow cooling had proved effective (Agarwal et al., 1981). Thus a further attempt with this procedure with a pool of Ig ε mRNA enriched fractions from a sucrose gradient (cf Figure 3-4, fractions 13 to 15) was carried out. The procedure was as described, (17) with cooling for 15 minutes in a 10 mL beaker of 90°C water. The resultant cDNA band only contained 3,400 cpm Cerenkov, indicating that secondary structure was not important or that its strength was such as not to be affected by the

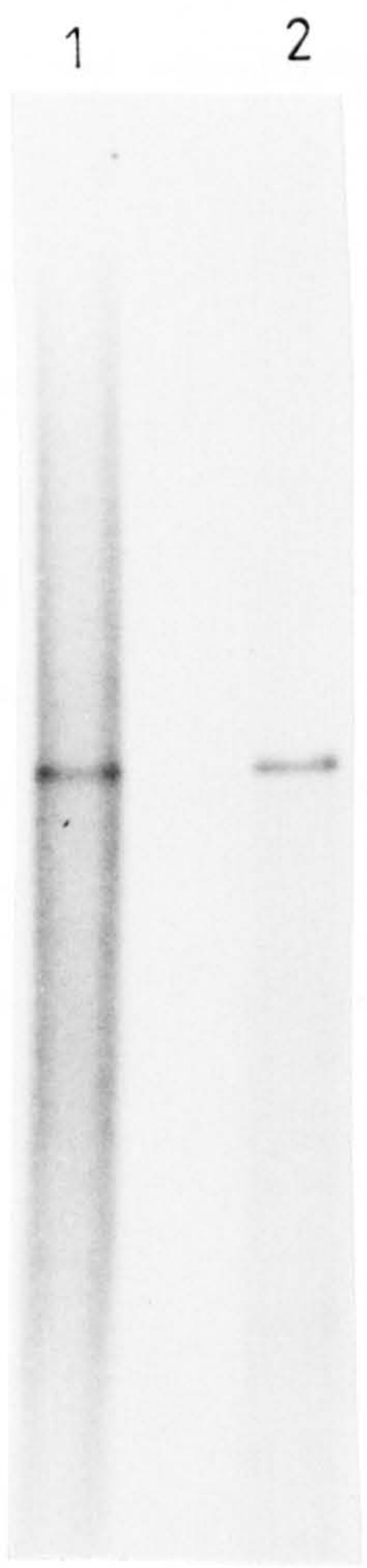


Figure 4.6 Transcription products of 266 BL mRNA, generated by oligodeoxynucleotide P6, labeled at its 5' end by [^{32}P] lane 2 and labeled by incorporation of [^{32}P] into the transcription products lane 1. The transcripts analyzed by alkaline agarose gel electrophoresis.

primers presence. The material was extracted from the gel slice and used for rescreening colonies positive for the internally labelled 1,700 base cDNA transcript, (Chapter 5). The inability of the conditions described by Agarwal et al (1981) to improve priming, resulted in reversion to the methods previously being used.

The correlation of translational activity with priming of cDNA transcripts has been used as a valuable indicator of the identity of primer specificity (Houghton et al., 1980a). This correlation of activities was demonstrated for gradient fractions shown in Figure 3-8, showing immunoprecipitation of Ig ε translational activity. The results of the priming reactions using 2% of each gradient fraction as used in the translation of the RNA, are shown in Figure 4-7. The bands from the gel were cut out and the (^{32}P) incorporation determined by scintillation counting, the results are shown below.

Gradient fraction	cpm - Background Gel Slice
11	310
12	730
13	780
14	350
15	130
16, 17, 18	Not determined

The results demonstrated a correlation with Ig ε activity (Figure 3-8). The incorporation of radioactive primer into the 1,700 base long cDNA transcript, indicated that sufficient incorporation could be obtained on scaling up. To scale up, 50% (25 µL) of sucrose gradient fraction 13 was used and products were

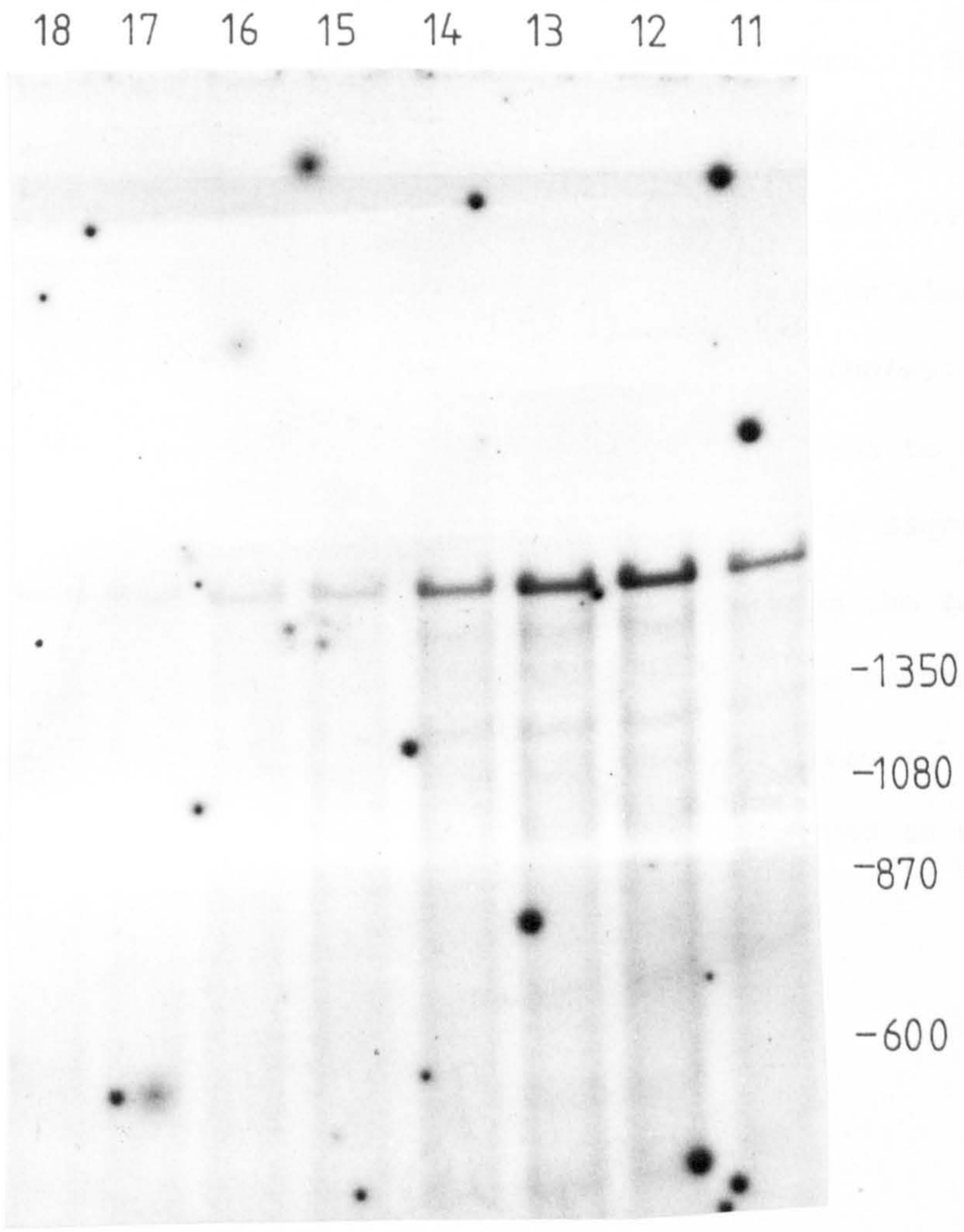


Figure 4.7 Transcription products of 266 BL mRNA, generated by oligodeoxynucleotide P6 on fraction from sucrose gradient 11 - 18 as shown in figure 3 - 8. [^{32}P]transcript analyzed by alkaline agarose gel electrophoresis.

run on an alkaline (1.2%) agarose gel. This lower percentage of agarose was chosen to enable a more efficient extraction. The incorporation into the 1,700 base cDNA was low (3,700 cpm) instead of an estimated value of approximately 8,000 cpm Cerenkov. This time it was decided to attempt sequencing with this number of counts, fortunately, after extraction, 3,220 cpm, Cerenkov was obtained. This small amount of radioactivity was subjected to 4 base cleavage reactions A>C, T+C and G for 30 minutes, and C for 40 minutes, the various steps being carried out carefully to reduce losses to a minimum. The sequencing digests were developed on an 8% sequencing gel, running the bromophenol blue dye marker to 2/3 from the top. The gel was autoradiographed for 4 weeks at -70°C. The sequence obtained (Figure 4-8 and 4-9) was that expected for primed Ig ε mRNA. From this sequence a 22 oligonucleotide probe was designed to screen transformed E. coli for cloned Ig ε mRNA sequences.

Figure 4.8 Nucleotide sequence analysis of Ig ε cDNA 5' -[^{32}P], labeled cDNA recovered from a gel was subjected to Maxam and Gilbert base-specific chemical cleavage reactions.

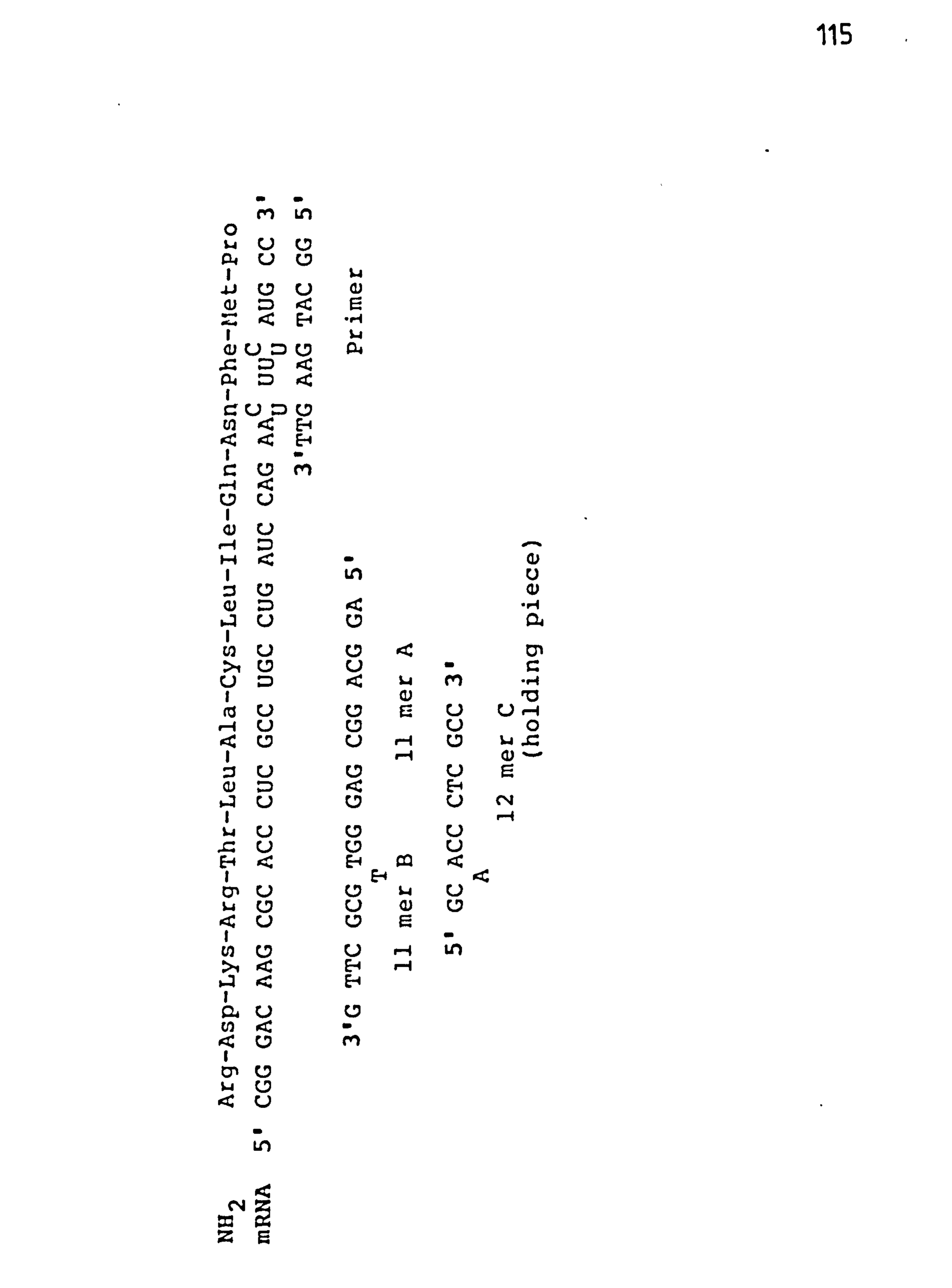


Figure 4.9 Nucleotide sequence obtained for the Ig ε cDNA, the primer used in cDNA synthesis, and the oligodeoxynucleotide probe consisting of two 11 and one 12 base long sequences constructed to the Ig ε cDNA sequence.

CHAPTER 5

Cloning of messenger RNA and Isolation of an Immunoglobulin Epsilon clone

The methods used are described in Chapter 2, the outline being as follows. The RNA used in attempts at cloning was partially purified poly A^+ RNA (Chapter 3). The initial attempts at synthesis of double stranded cDNA (2s cDNA) were using method (20) derived from a combination of methods described by Buell et al., (1978) and Monahan et al., (1976). The last attempt was using method (22), as described by Gough et al., (1980). The 2s cDNA produced after SI treatment was followed by DNA polymerase I treatment, attachment of Hind III linkers and ligation into Hind III digested, bacterial alkaline phosphatase treated pAT 153 (Fiddes and Goodman, 1979) (23). This ligation mix was then used to transform competent E. coli, MRC8 (GMAG note 9, supplement 1) (28). The transformants were selected on nutrient broth agar plates supplemented with (100-200 µg/mL) carbenicillin, N-acetyl D glucosamine (200 µg/mL) and D, L,α ε-diamino-pimelic acid (50 µg/mL) (24). The clones were screened with an oligodeoxynucleotide probe.

Initial results with cloning proved unsatisfactory, the outline of these results is given below. The initial results for 2s cDNA synthesis with poly A^+ RNA enriched for λ light chain proved successful producing 2s cDNA of the expected size range $\sim$1000 base pairs (20). The application of the protocol to Ig ε enriched fraction 17, from sucrose gradient as in Figure 3-5 and 3-6, did not prove satisfactory (see Figure 5-1). The molecular size of the 2s

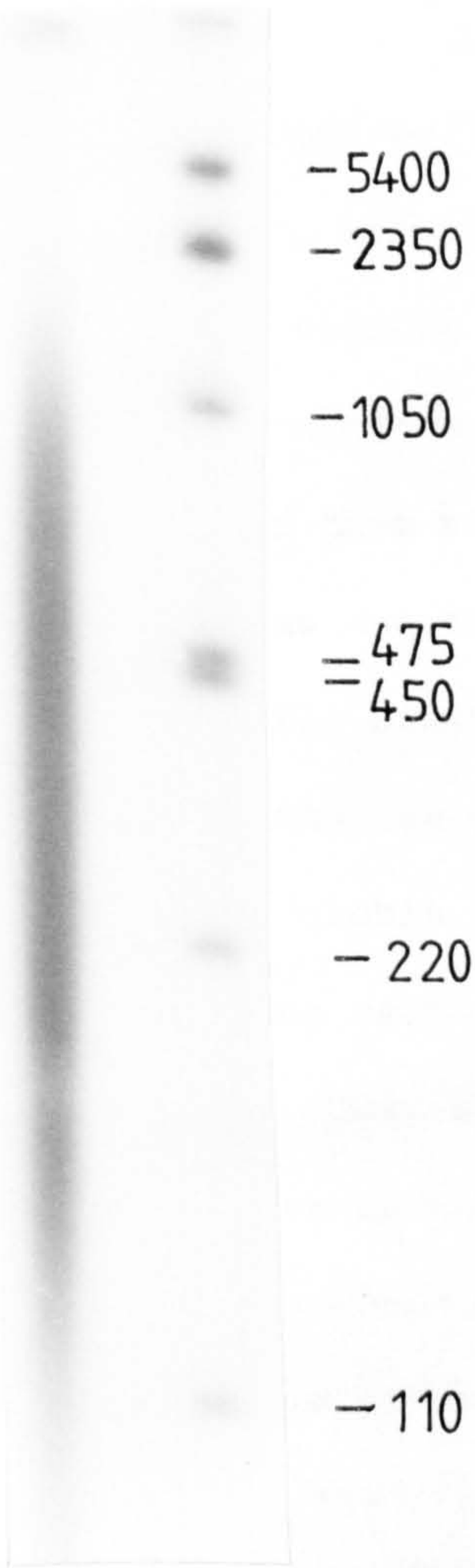


Figure 5.1 Autoradiograph of a size analysis of [^{32}P] labeled double stranded cDNA made from poly A+ RNA enriched in Ig ε translation activity (lane 1). Lane 2 shows the Hind III digest of phage PM2 DNA containing the fragments of lengths (in base pairs) as indicated.

cDNA after SI treatment and DNA polymerase I treatment was below the expected optimum of 1,000-2,000 base pairs. The 2s cDNA at sizes >1000 base pairs was electroeluted into DE52, eluted from the DE52 and precipitated, linkered and transformed into E. coli MRC8. The obtained transformant colonies were then subjected to screening with oligodeoxynucleotide primers P6 and P8 ((see Chapter 4), 11 and 14 bases long respectively) (30). The results (Figure 5-2) using P8 with recombinant clones immobilized on nitrocellulose (Grunstein and Hogness, 1975) suggested that many of them may be positive clones, but rescreening demonstrated that this was due to variation in size of the colonies as a result of nonspecific binding. At this time Wallace et al., (1981) demonstrated the use of synthetic 14 base oligodeoxynucleotide (14 dN Mers) on globin gene containing colonies. They used a different colony screening method similar to Gergen et al., (1979), providing lower backgrounds. This observation was surprising since the method tends to give higher backgrounds on the filters, as described by Gergen et al., (1979). These backgrounds on filters were found for the oligonucleotides as probes as in Figure 5-3, but the background from the colonies was significantly lower (31). This lower colony background probably results from the method of lysis, as the majority of the bacterial debris is washed off by the NaOH lysis and subsequent washes. The use of this method did provide some colonies with slightly higher signals than others but not as convincingly different as expected from the data of Wallace et al., (1981). These colonies were pooled and subjected to two further screens. First, plasmid DNA was purified by the alkaline method (26),subjected to electrophoresis in a 1.4% TAE gel, and transferred

Figure 5.2 Autoradiograph of a colony hybridization using oligodeoxynucleotide P8 labeled with [^{32}P] to clones obtained from 266 BL cDNA inserted at the Hind III site of pAT153. The colonies were grown up on and lysed *in situ* on nitrocellulose filters, overlaid on L-agar plates supplemented with 100µg/ml carbenicillin.

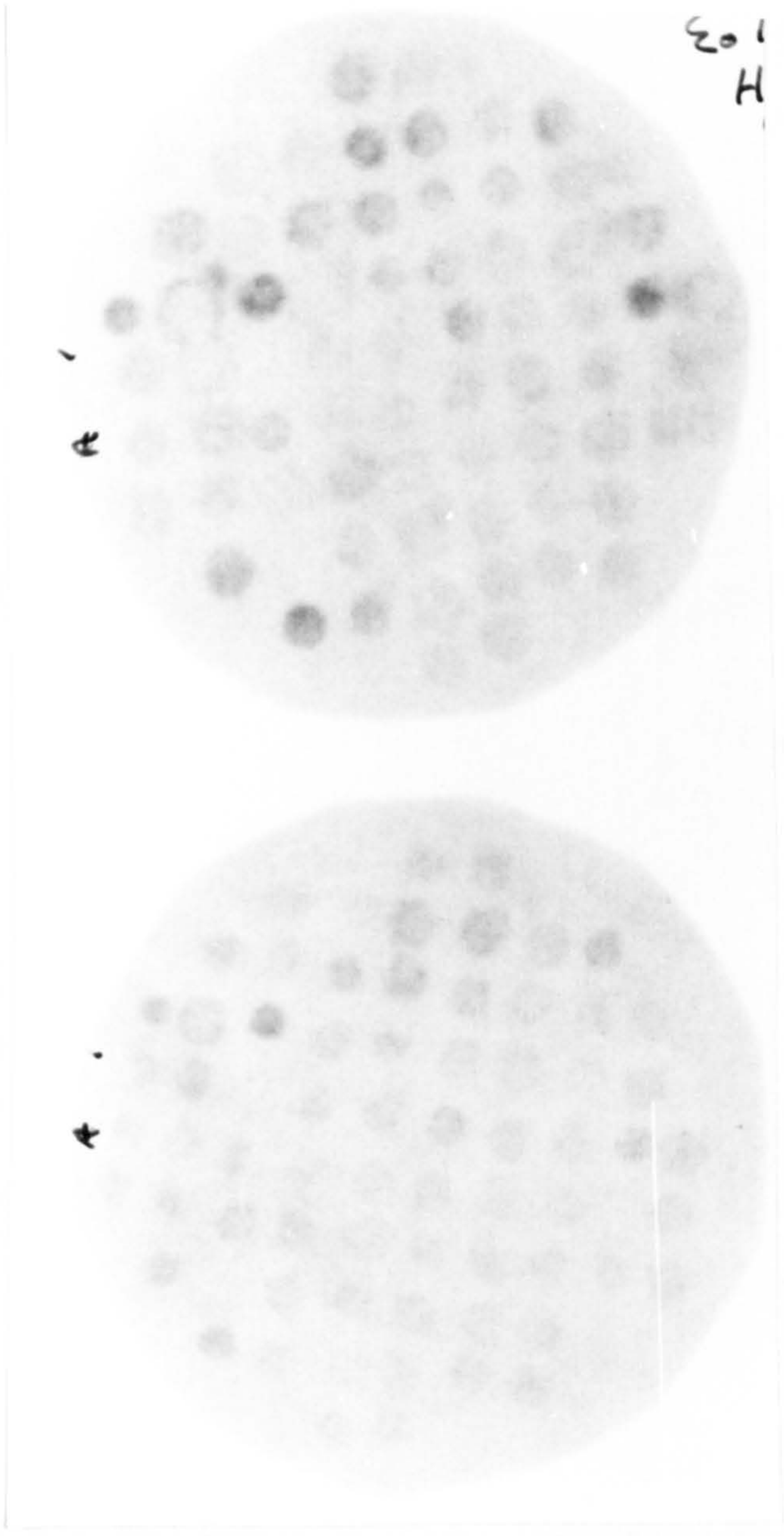


Figure 5.3 Autoradiograph of a colony hybridization using oligodeoxynucleotide P8 labeled with [^{32}P] to clones obtained from 266 BL cDNA inserted at the Hind III site of pAT153. The colonies were grown up on L-agar plates supplemented with 100µg/ml carbenicillin, lifted off onto Whatman 541 filters and lysed *in situ*.

to nitrocellulose by the Southern (1979) procedure (18). This DNA blot was then hybridized to primer P8, as hybridization to partially purified DNA is known to give significant results at 11 base oligodeoxynucleotide level (Suggs et al., 1981 (34)). The result is shown in Figure 5-4, indicating that none of the colonies contain sequences of the Ig ε gene, with E. coli chromosomal DNA giving a stronger signal due to its higher complexity resulting in sequences close to P8 being present. Second, a cDNA hybridization probe was prepared using (α^{32}P) dATP to internally label a specifically primed cDNA using oligodeoxynucleotide P6 as described in Chapter 4, Figure 4-6. This was prepared because, although the conditions for hybridization of long nucleotides are known (Southern 1979), the conditions to be used for oligonucleotide hybridization were not fully established. The probe was used to screen colonies immobilised onto nitrocellulose. The result is shown in Figure 5-5A, indicating a number of positive colonies. These were pooled and rescreened with 3,400 Cerenkov cpm of 5' labelled cDNA. This was done, as there was significant incorporation in the (α^{32}) dATP labelled primed cDNA's, which was not due to specific priming events, as it was not found for end labelled primer. This would have resulted in a probe with significant contamination of non specific sequences, a probe present at 0.5% of total counts giving results in this type of screening (Gergen et al., 1979).

The result from this colony hybridization is shown in Figure 5-5B. The colonies giving the strongest result were subjected to Maxam and Gilbert (1977) sequencing from the Hind III ends of the

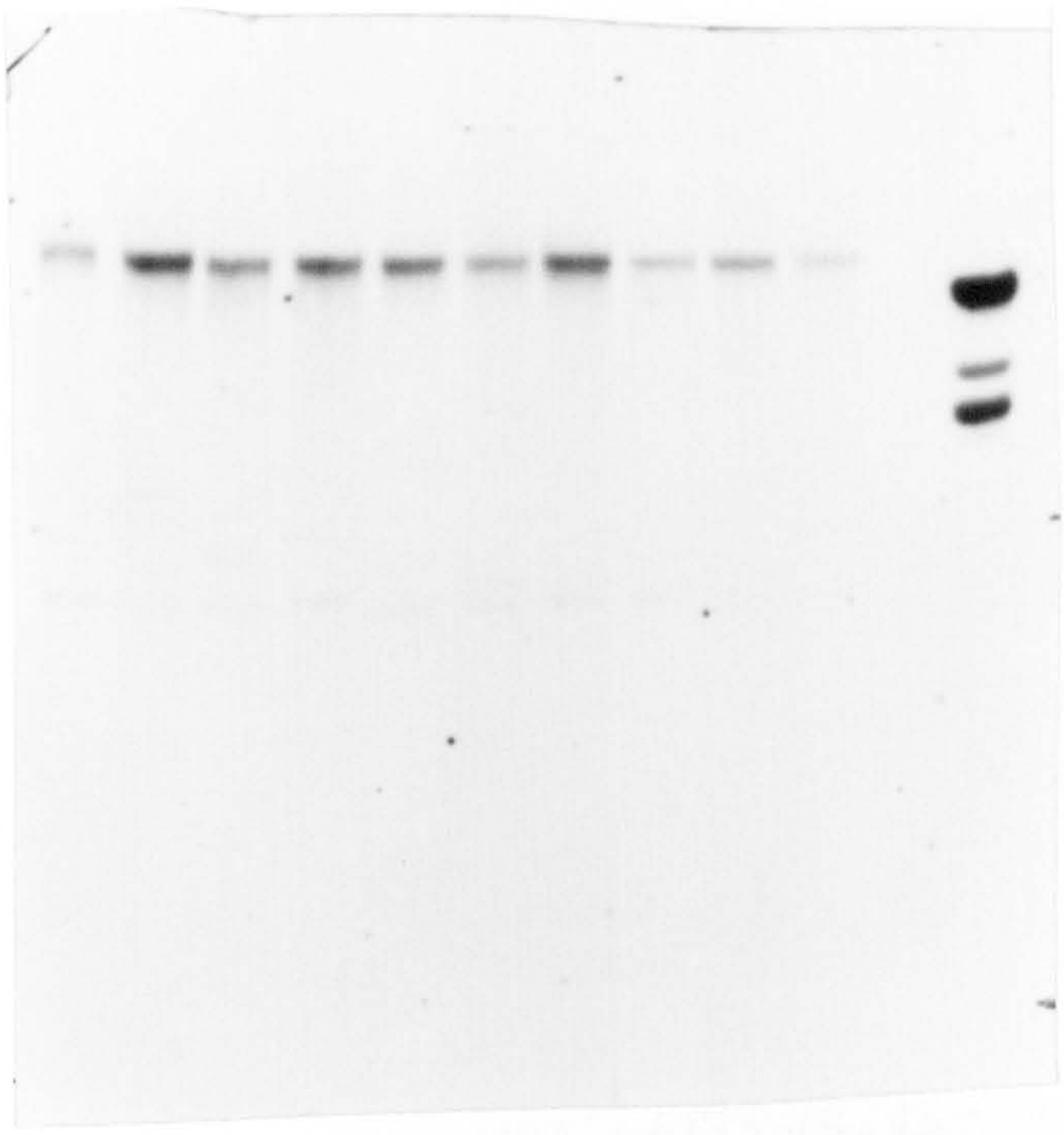
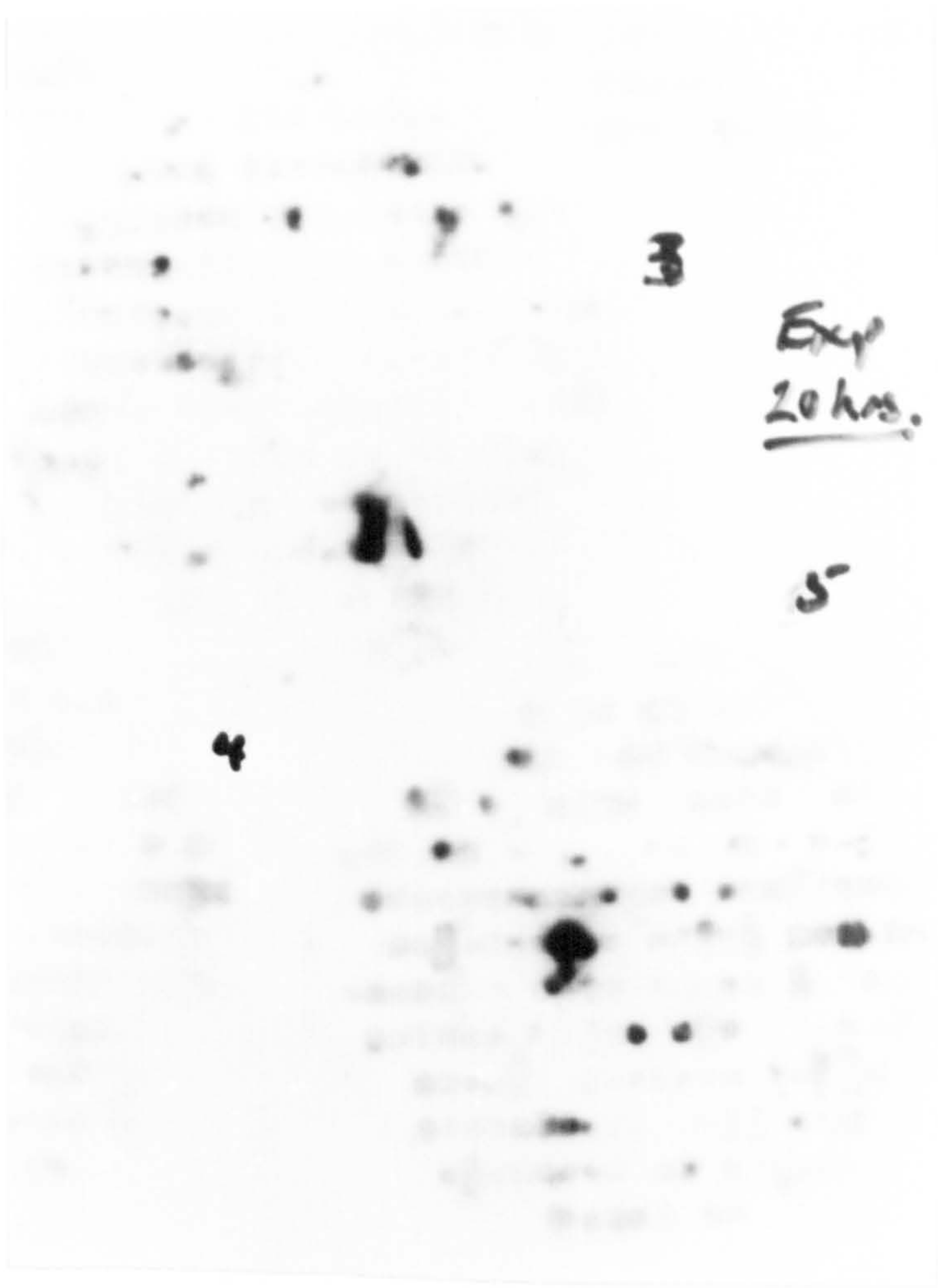


Figure 5.4 Autoradiograph of a filter hybridization between partially purified E. coli plasmid DNA fractionated on a 1.4% agarose gel and [^{32}P] labeled oligodeoxynucleotide P8.

A



B

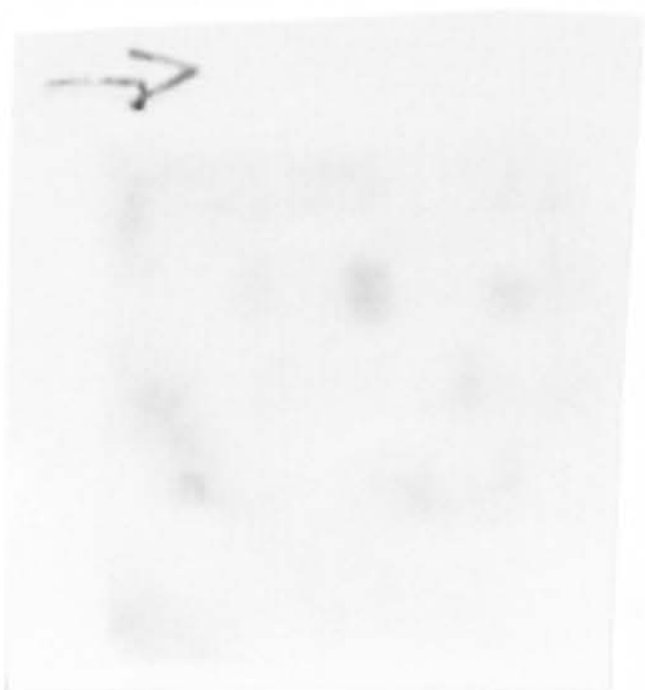


Figure 5.5 Autoradiograph of colony hybridizations.

A. Colonies hybridized with an internally [^{32}P] labeled cDNA generated by oligodeoxynucleotide P6 on 266 BL poly A+ RNA.

B. Colonies positive in (A) hybridized with a cDNA generated by a 5' [^{32}P] labeled oligodeoxynucleotide, P6 on 266 BL poly A+ RNA.

inserted sequence (35). This demonstrated that the two cloned Hind III fragments ∿110 and 150 base pairs long were not of Ig ε mRNA origin. The size of the inserts was also smaller than expected on the basis of the material selected for cloning of >1000 base pairs long. The origin and nature of these clones is uncertain.

A new recombinant library was constructed from RNA purified on the 15% vs 32% sucrose isokinetic gradient, as in Figures 3-7 and 3-8. The use of E. coli DNA polymerase I for 2nd strand synthesis was tried in view of the success with this method for both mouse and human immunoglobulin mRNA cloning (Gough et al., 1980; Molgaard personal communication) (21). The results for ls cDNA with RNA from this gradient proved the quality of the RNA and the ability of the lst strand synthesis to produce the expected profile of cDNA. Initial attempts with E. coli DNA polymerase I following the method described by Wickens et al., (1978) method (21), are shown in Figure 5-6, the first strand synthesis was as expected, but the 2nd strand synthesis was incomplete for fraction 16, but with fraction 18 large amounts of a product ∿1400 bases long was produced. To improve on this potentially incomplete synthesis the salt concentration (KCl) was lowered from 134 mM to 103 mM, and the E. coli DNA polymerase I increased from 17 u/mL to 120 u/mL in the second strand reaction.

This was tried as the amount of enzyme and salt concentration were demonstrated to be important in determining the production of full length double strands (Wickens et al., 1978), the salt concentration being well within the range to prevent nonspecific polymers. This resulted (Figure 5-7) in what appeared to be

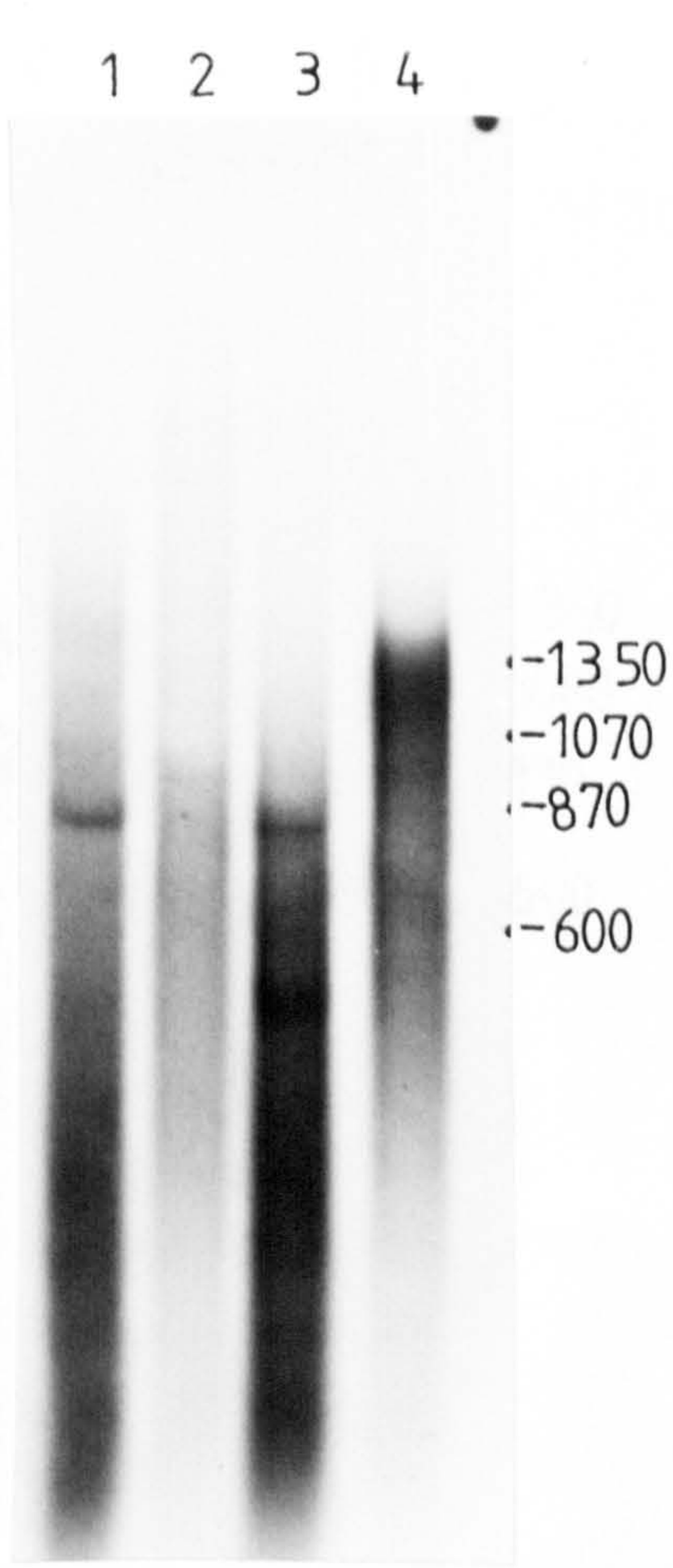


Figure 5.6 Autoradiograph of cDNA synthesised to 266 BL poly A+ RNA fractionated on a sucrose gradient as in Figure 3 - 7 and 3 - 8. First strand cDNA (lanes 1 and 3) and second strand cDNA (lanes 2 and 4) from sucrose gradient fractions 16 (lanes 1 and 2) and 18 (lanes 3 and 4). Sizes indicated in bases.

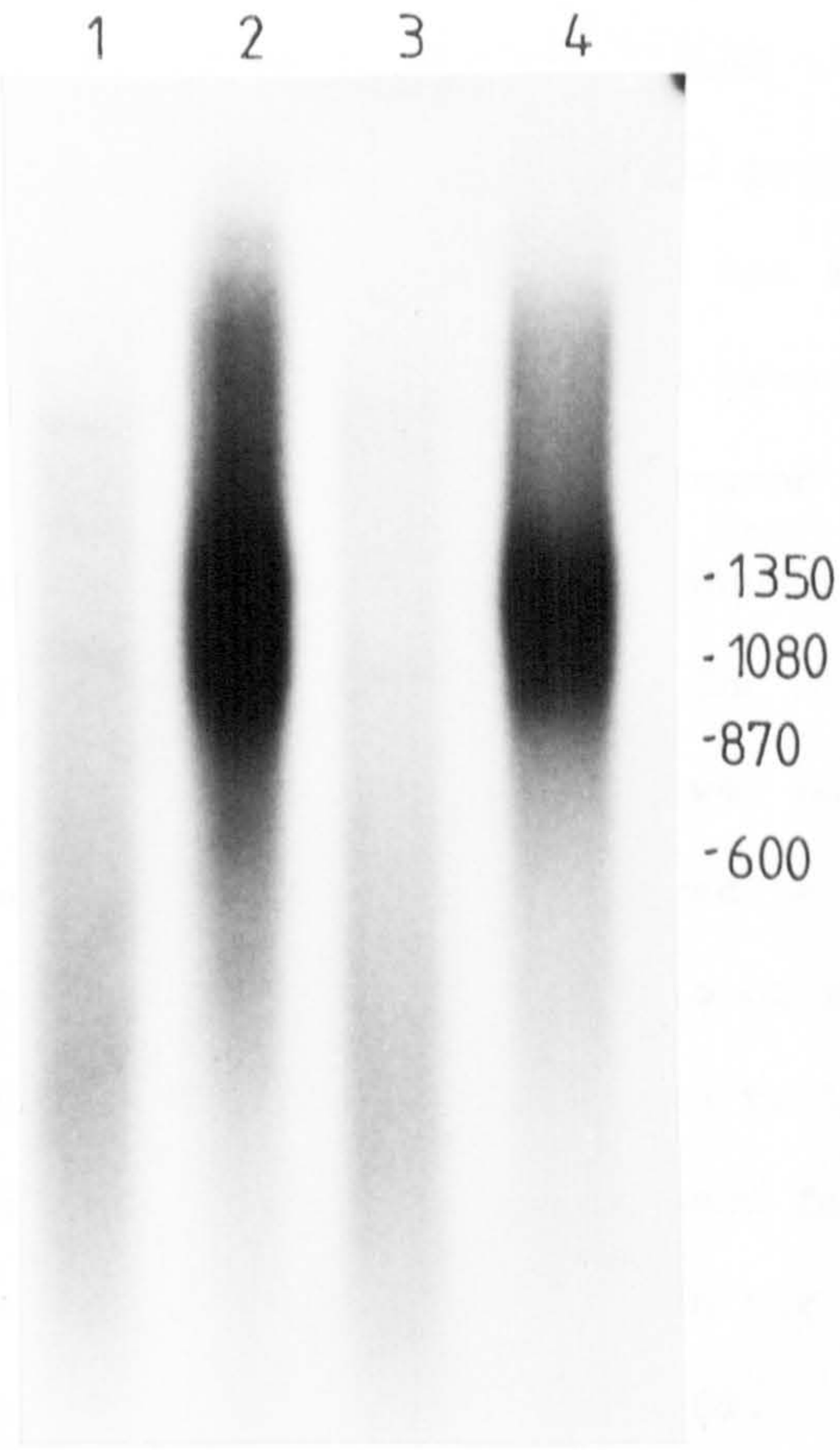


Figure 5.7 Autoradiograph of cDNA synthesised to 266 BL poly A+ RNA fractionated on a sucrose gradient as in Figure 3-7 and 3-8. First strand cDNA (lanes 1 and 3) and second strand cDNA (lanes 2 and 4), from sucrose gradient fractions 14 (lanes 1 and 2) and 12 (lanes 3 and 4). Sizes indicated in bases.

adequate 2nd strand synthesis, but also with the generation of a large amount of apparently non specific material at ∿1000 bases which, judging by the radioactivity, represented a large degree of de novo synthesis in the 2nd strand reaction.

These results led us to use the method (22) (Materials and Methods, Gough et al., 1980), as this had previously been used successfully without production of these non specific products (Molgaard personal communication). The results are shown in Figure 5-8 A and B. These demonstrated good second strand synthesis with long 2nd strand transcripts present as bands. The presence of apparently non specific material in the 2nd strand reaction was also found, but at the size of ∿600 bases it was not considered a problem. The use of 3 units of SI nuclease appeared to have only partially removed the hairpin, in view of the ratio of single:double strand activity 50 units was tested and appeared to be adequate. This method (3) (Materials and Methods) was used for the synthesis of double stranded cDNA on the peak fraction for Ig ε mRNA from the sucrose gradient (fraction 12, Figure 3-8). The results with 1st strand synthesis (Figure 5-7) indicated that about 200 ng of 1s cDNA was synthesised per µL of this fraction, 10 µL, was used for gene synthesis, resulting in production of 0.8 µg of cDNA. The results for gene synthesis are shown in Figure 5-9. These SI nuclease treated double stranded cDNAs ('genes'), were subjected to fractionation by Bio-Gel A150-M (Mesh 100-200, Bio-Rad Ltd) column chromatography. The result is shown in Figure 5-10, indicating the amount and size distribution of the 'genes'. Fractions 31-47

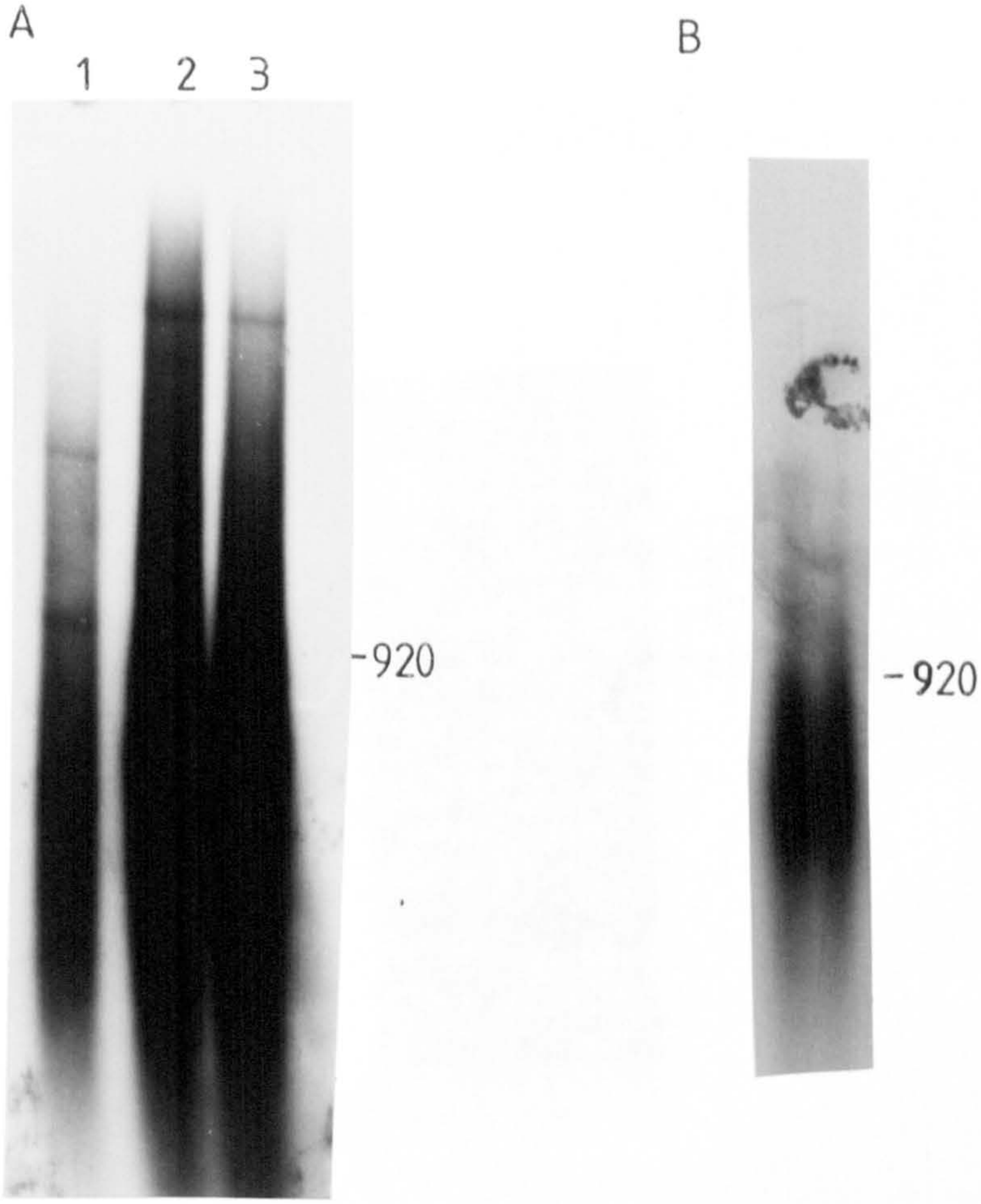


Figure 5.8 Autoradiograph of cDNA synthesised to 266 BL poly A+ RNA fractionated on a sucrose gradient as in Figure 3-7 and 3.8 using fraction 14.

A. First strand cDNA (lane 1), second strand cDNA (lane 2), S_1 nuclease treatment of second strand cDNA (lane 3).

B. Further S_1 nuclease treatment of second strand cDNA as in A, lane 2. Size indicated in bases from growth hormone clone.

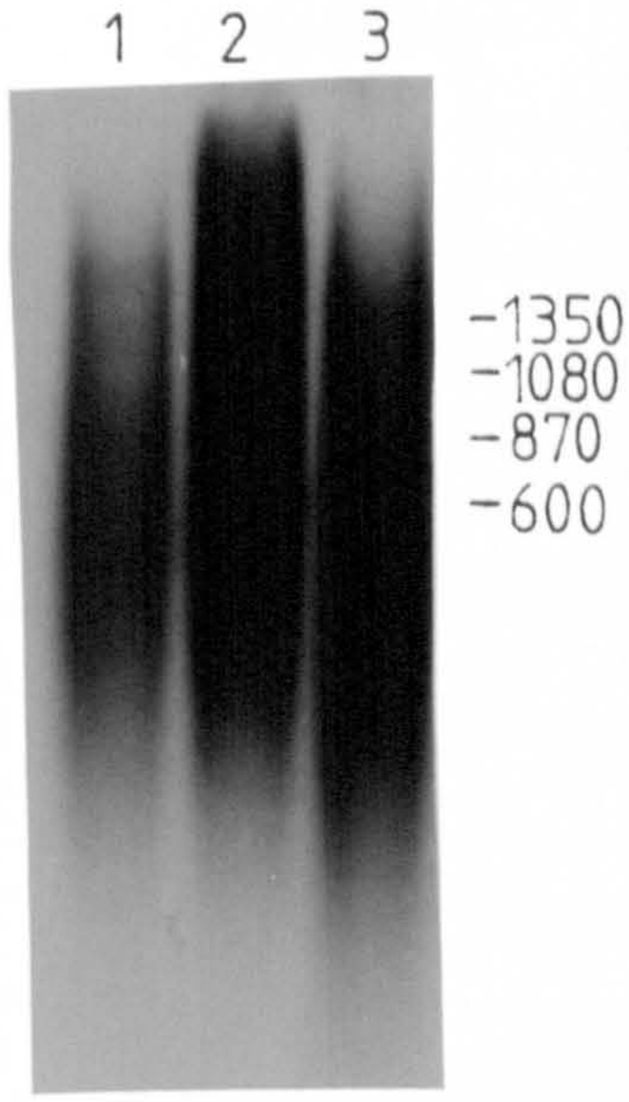


Figure 5.9 Autoradiograph of cDNA synthesised to 266 BL poly A+ RNA fraction 12 from sucrose gradient as in figure 3-7 and 3-8. First strand cDNA (lane 1), 2nd strand cDNA (lane 2), S_1 nuclease treated 2nd strand cDNA (lane 3). Sizes indicated in bases.

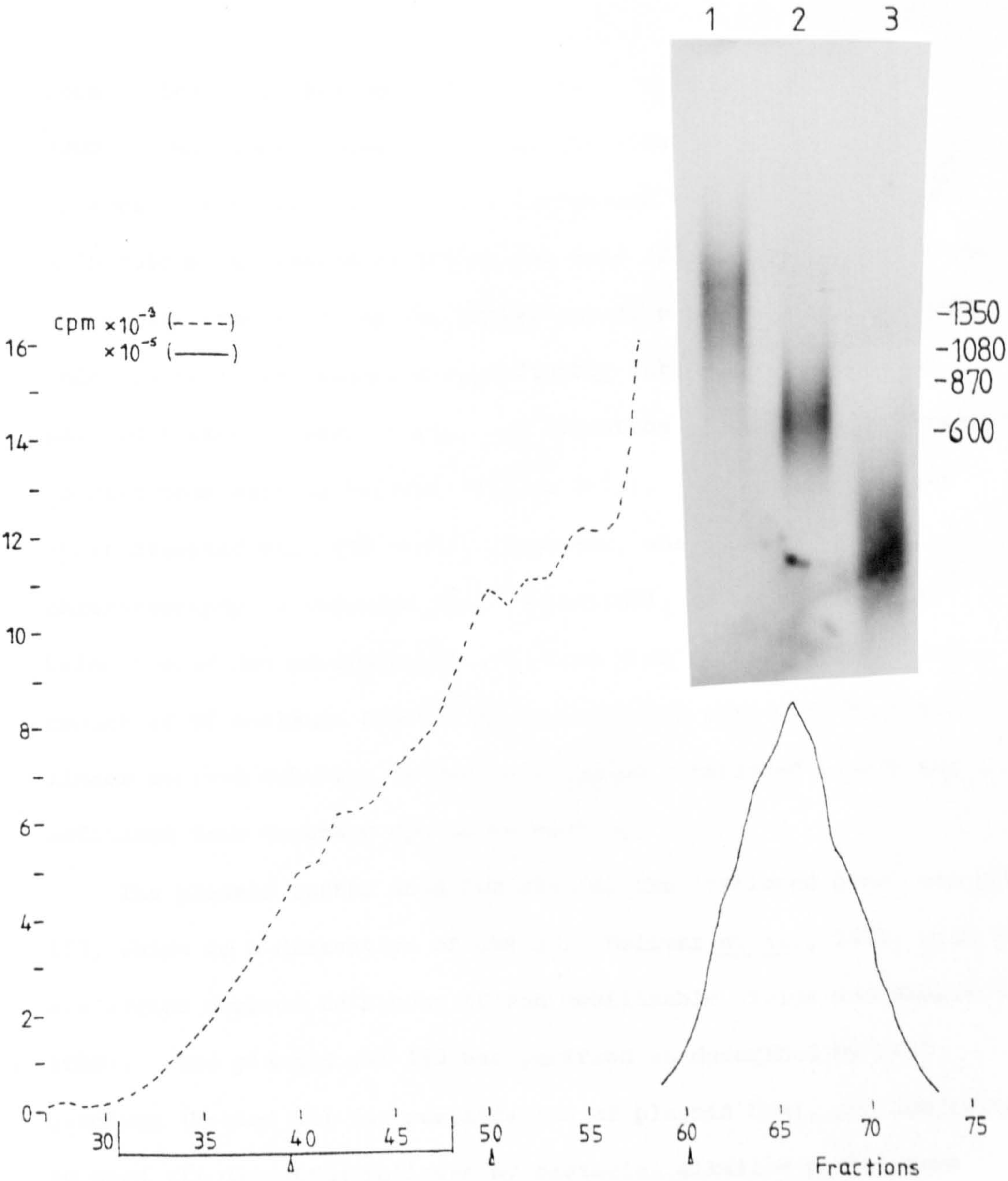


Figure 5.10 Fractionation of double stranded cDNA by Bio-Gel A150-M. The graph shows the elution profile of the cDNA determined from the Cerenkov counts. Fractions and pools of fractions indicated by arrows were analysed by alkaline gel electrophoresis as shown in the inset: fraction(s) 31 - 47 (lane 1), 50 (lane 2) and 60 (lane 3). Sizes indicated in bases.

were pooled (size average ∿1400 bases). This pool of 'genes' ≃200 ng, was then subjected to ligation with synthetic Hind III linkers, previously tested for ligation and cutting (Figure 5-11), a 75 fold molar excess of linker was used (23). This ligation and subsequent removal of excess linker was to provide Hind III sticky ends, suitable for ligation specifically into Hind III digested plasmid vector. The ligation and digestion of the linkers appeared to have been very successful (Figure 5-12). The removal of the short digested Hind III linker fragments, was achieved by chromatography on Sephadex G150 (superfine), the excluded peak being pooled and precipitated with tRNA (DNA free, yeast). The amount of SI nuclease treated double stranded cDNA with Hind III linker derived cohesive sequences attached ('tailored gene') was estimated from Cerenkov cpm to be ∿130 ng.

The plasmid vector used for cloning the 'tailored gene' was pAT 153, which is a derivative of pBR 322 (Bolivar et al., 1977) with the nic region removed to render it non mobilizable (Twigg and Sherratt, 1980). The plasmid pAT 153 was purified as described by CsCl gradient (Method(25) for purification of plasmid DNA), and subjected to Hind III digestion followed by bacterial alkaline phosphatase treatment (29). This results in the pAT 153 being unable to self ligate without the 5' phosphate. The treated pAT 153 was tested to determine the level of the background ligations, and also to test the integrity of the Hind III ends by a ligation in the presence of polynucleotide kinase (replacing the 5' phosphate and allowing regliation). The religation of the plasmid was determined by its

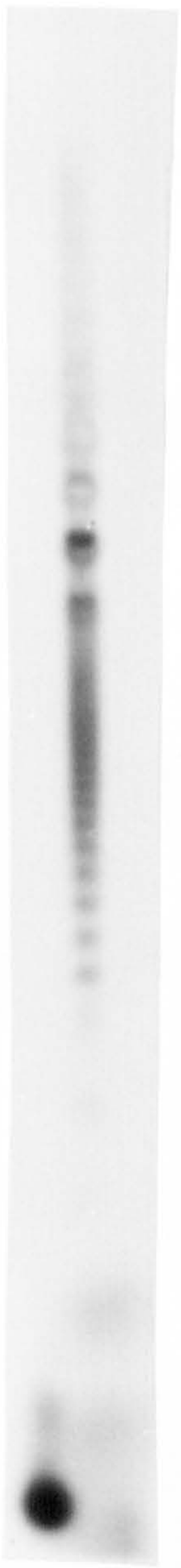


Figure 5.11 Test ligation of Hind III linkers. Electrophoresis and autoradiography of 5' [^{32}P] labeled linkers (lane 1), ligated (lane 2), Hind III digested after ligation (lane 3).

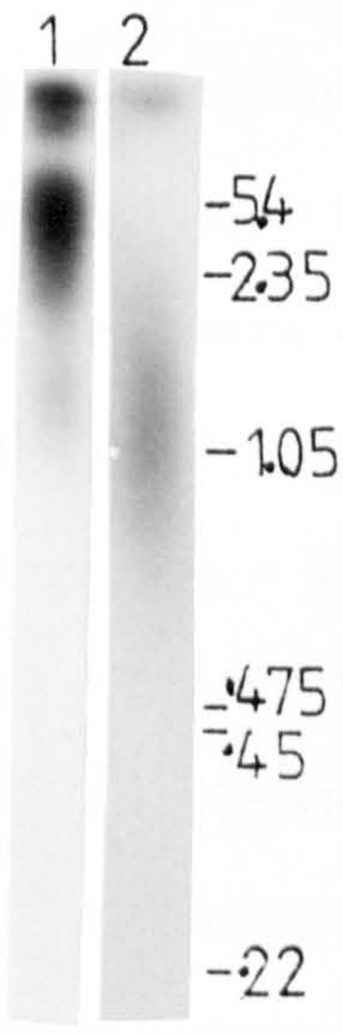


Figure 5.12 Ligation of Hind III linkers to cDNA. Electrophoresis and autoradiography of [^{32}P] labeled 266 BL cDNA ligated to Hind III linkers. Ligation of Hind III linkers to fractionated cDNA (lane 1); Hind III digest of the cDNA following ligation with Hind III linkers (lane 2). Sizes indicated in bases 10^{-3}.

transformation efficiency for background religation, the efficiency was 5 x 10^2 transformants/μg of DNA with the overall efficiency for supercoiled pAT 153 at 4.5 x 10^5 transformants/μg of DNA. The result of the ligation with replacement of the 5' phosphate was 9 x 10^5 transformants/μg, with the overall efficiency for supercoiled pAT 153 of 9 x 10^5 transformants/μg. This clearly demonstrated the reduced transformation frequency after Hind III digestion and subsequent removal of 5' phosphates, was not due to any nuclease degradation of the Hind III cohesive ends. The 'tailored gene' was ligated to the above prepared pAT 153 vector, (∿3 ng was ligated with 100 ng of pAT 153, (approximately a 30 fold molar excess of plasmid)) for 10 hrs at 15°C, and the reaction mix used to transform MRC8. The transformation efficiency as judged by supercoiled pAT 153 was 4.4 x 10^5 transformants/μg, 465 transformants were obtained for the 'tailored gene', with 45 transformants in its absence as a control. This represents ∿125 recombinants/ng of 'tailored gene'. This was repeated and resulted in 320 transformants with 'tailored gene' (28, 29).

The screening of these recombinant bacteria was done using a 22 base oligodeoxynucleotide synthesised to the previously determined portion of the Ig ε mRNA sequence as in Figure 4-9, which also shows an error made in the design of the probe. The 22 base oligodeoxy-nucleotide was synthesised in two sequences of 11 nucleotides, with a 12 nucleotide complementary sequence being used to effect the subsequent ligation of the two halves of the 22 base oligodeoxy-nucleotide probe (33). These sequences were synthesised by the

Chemistry Dept., of G. D. Searle. To test the specificity for the ligation of oligonucleotides, various combinations of labelled and unlabelled oligonucleotides were ligated together. This is shown in detail in Figure 5-13. The overall result showed that the expected ligations occurred efficiently with some side reactions.

In the labelling of material for use in probing colonies, only 11 base oligodeoxynucleotide B was phosphorylated with polynucleotide kinase, as the oligonucleotides were synthesised without the 5' phosphate. This diminishes the probability of side reactions occurring and also enables the production of a probe of high specific activity. Phosphorylation of oligonucleotide B using >5000 Ci/mmol (γ^{32}P) ATP with polynucleotide kinase results in phosphorylation at >5000 Ci/mmol. In the subsequent ligation to oligonucleotide A in the presence of oligonucleotide C, only ligations with phosphorylated oligonucleotide B will take place resulting in production of the 22 base oligonucleotide probe at >5000 Ci/mmol. The 22 base oligonucleotide probe was prepared using 100 µCi of >5000 Ci/mmol (γ^{32}P) ATP to label oligonucleotide B. The subsequent 22 base oligonucleotide was purified after electrophoresis on a 15% polyacrylamide gel in 7M urea, TBE. The recovery from electroelution into DE52 (Whatman) was found to be 70%. The 22 base oligonucleotide probe was also subjected to Maxam and Gilbert sequencing (1977). This partial sequencing showed nearly all the sequence was correct (33, 35).

The screening of colonies was done essentially was described by Wallace et al., (1981) see Methods. In brief, the transformants

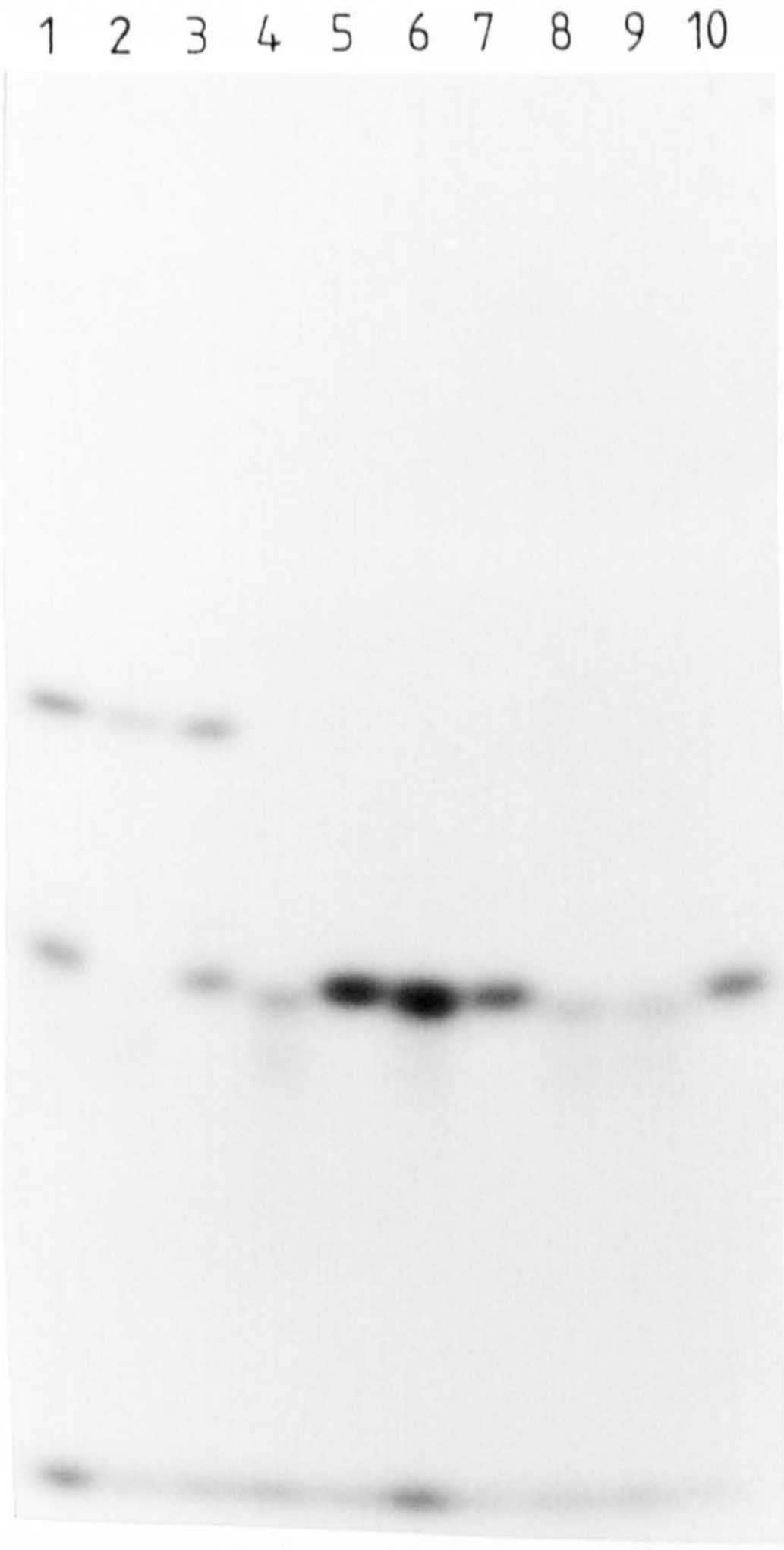


Figure 5.13 Electrophoresis and autoradiography of 5' [^{32}P] labeled oligodeoxynucleotides A, B and C used for construction of a hybridization probe (see Figure 4.9). All ligations containing A and B contain A with the 5' PO_4 added, all ligations containing C contain it without a 5' PO_4. 5' [^{32}P] labeled A and B are indicated A* or B*.

Lane 1; A*, B* and C : lane 2; A*, B and C : lane 3; A, B* and C : lane 4; A* and C : lane 5; B* and C : lane 6; A* and B* : lane 7; A and B* : lane 8; A* and C : lane 9; A* : lane 10, B*. Length indicates in bases.

were lifted onto Whatman 541 filter papers and amplified on chloramphenicol agar plates. The colonies on the filters were then lysed, air dried and used for hybridization. The plates after lifting the colonies were grown for a few hours at 37°C to enable selection of the positive colonies from the initial transformants (31).

Initially the colonies were hybridized at 55°C in 3 x SSC overnight, with washing in 6 x SSC firstly at 0°C and then briefly at 55°C. The results are shown in Figure 5-14A, the colonies themselves being easily visualized using ethidium bromide staining as shown in Figure 5-14B. This result showed a high background of binding to the filter paper. This was reduced to a low level by reduction in the amount of 22 base probe used from 1 x 10^7 to 1.0-0.3 x 10^6 cpm/ml, these conditions were used in subsequent hybridizations. The ability of the probe to hybridize at higher temperatures was investigated by carrying out hybridizations at temperatures up to 75°C as described. This demonstrated that the 22 base probe with its mismatch was capable of hybridization up to 70°C (Figure 5-15). As a result of screening the transformants the 5 colonies giving a positive result were streaked out to single colonies and subjected to a further screen. This was to ensure that a single clone was selected before further analysis.

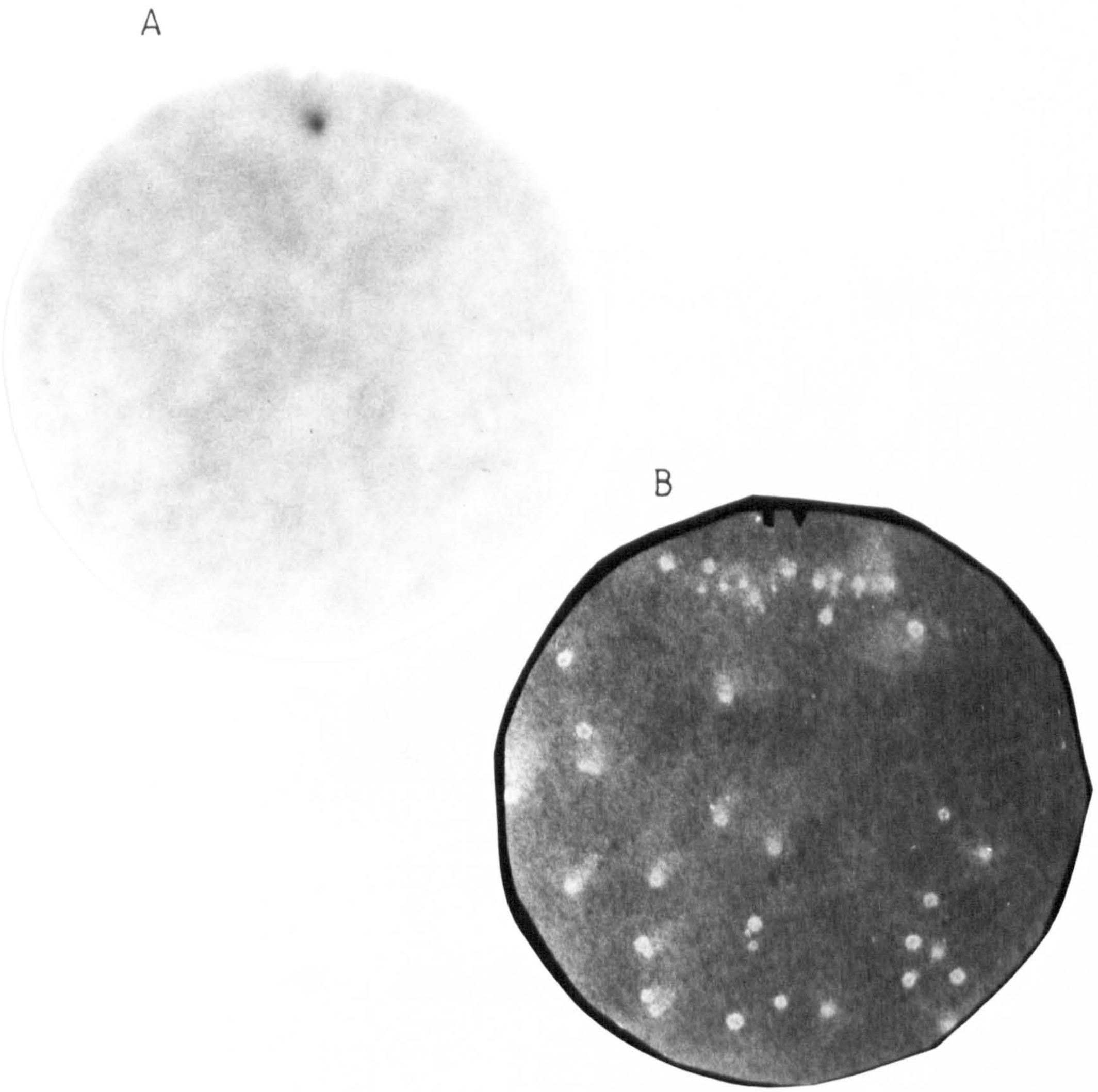


Figure 5.14 Autoradiograph of the [^{32}P] labeled 22 base oligodeoxynucleotide probe hybridized to 266 BL poly A+ RNA derived clones and photograph of ethidium bromide stained clones.

A. Colonies lifted from L-agar plates supplemented with 100 µg/ml carbenicillin lysed *in situ* onto Whatman 541 filters and then hybridized to the oligodeoxynucleotide probe overnight. Filter exposed for 2 hours at room temperature.

B. As above but after exposure the filter was stained with 100 µg/ml ethidium bromide to show other colonies not hybridizing with the oligodeoxynucleotide probe.

Figure 5.15 Autoradiograph of the [^{32}P] labeled 22 base oligodeoxynucleotide probe hybridized to an array of Ig ε containing clones, interspersed with -ve clones, at differing temperatures overnight. The washing conditions were as described in methods for all hybridization temperatures. TM appears to be ~70°C.

CHAPTER 6

Characterisation of Immunoglobulin Epsilon Clone

The five clones which were initially isolated were subjected to analysis of their cloned insert size. Quick-boiling lysis preparations were made of plasmid DNA and then subjected to Hind III digestion. The products were analysed on a TBE 1.4% agarose gel and the clone with the largest insert of approximately 2 kilobases (Kb) pJJ71 was characterised further. Further transformants were generated and six further positive clones from 22 oligonucleotide probing were analysed, but none contained an insert size of greater than 2Kb.

The plasmid pJJ71 was prepared in sufficient amounts for sequence analysis. A 1 litre culture was grown up for this and DNA prepared by the cleared lysate method followed by CsCl gradient purification (25). Any residual tRNA was removed on a Sephacryl S300 column the excluded peak yielding 110 µg of DNA. Initial restriction data was determined from DNA obtained by the quick-boiling lysis method (27). The strategy employed was to subject the DNA to a series of digestions, followed by gel electrophoresis. These gels were then transferred to nitrocellulose by the method of Southern and probed using the 22 oligonucleotide probe. (18,34). This procedure led to the determination of restriction sites and their orientation, as the 22 oligonucleotide probe hybridizes near to the 3' end of the Ig ε mRNA clone. The results for various digests and the equivalent Southern blots is shown (Figure 6-1). This resulted in the construction of an initial restriction map (Figure 6-2). The exact positions of the sites were uncertain from

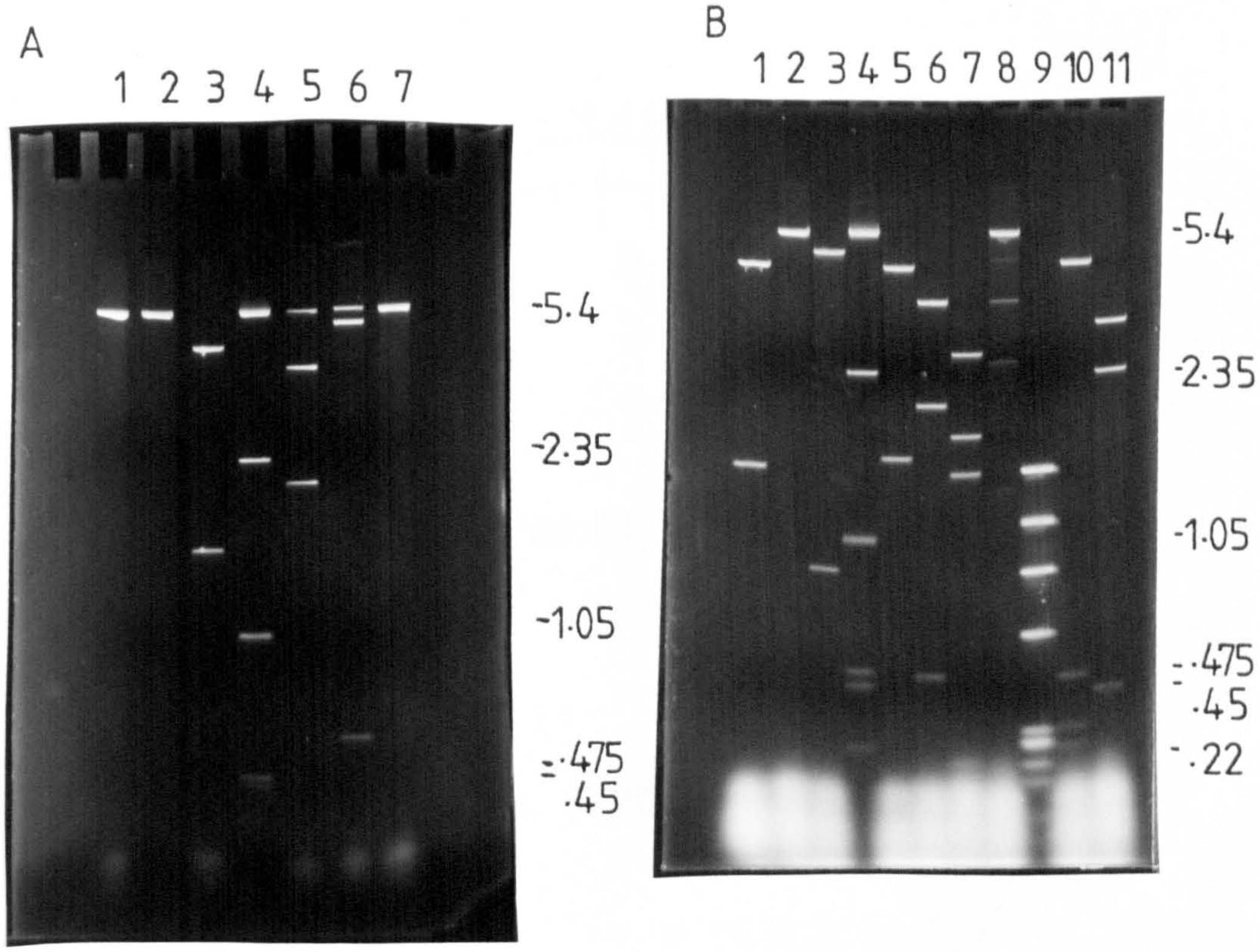


Figure 6.1 Restriction digests on pJJ71 and electrophoresis with Southern transfer of restricted DNA, hybridized with the 22 oligodeoxynucleotide. The autoradiograph of the Southern hybridization is shown in the overlay.

A. lane 1 Pst I; lane 2 EcoRI; lane 3 Sal I; lane 4 Marker, Hind III PM2; lane 5 Hind III; lane 6 Bam HI; lane 7 Pvu II.

B. lane 1 Pst I, Hpa I; lane 2 Pst I; lane 3 Pst I, Pvu II; lane 4 Marker, Hind III PM2; lane 5 Pst I, Sma I; lane 6 Pst I, Bgl II; lane 7 Bgl I; lane 8 Bal I; lane 9 Marker, Hae III øX 174; lane 10 Bst E II; lane 11 Ava I. Sizes indicated in base pairs x 10^{-3}.

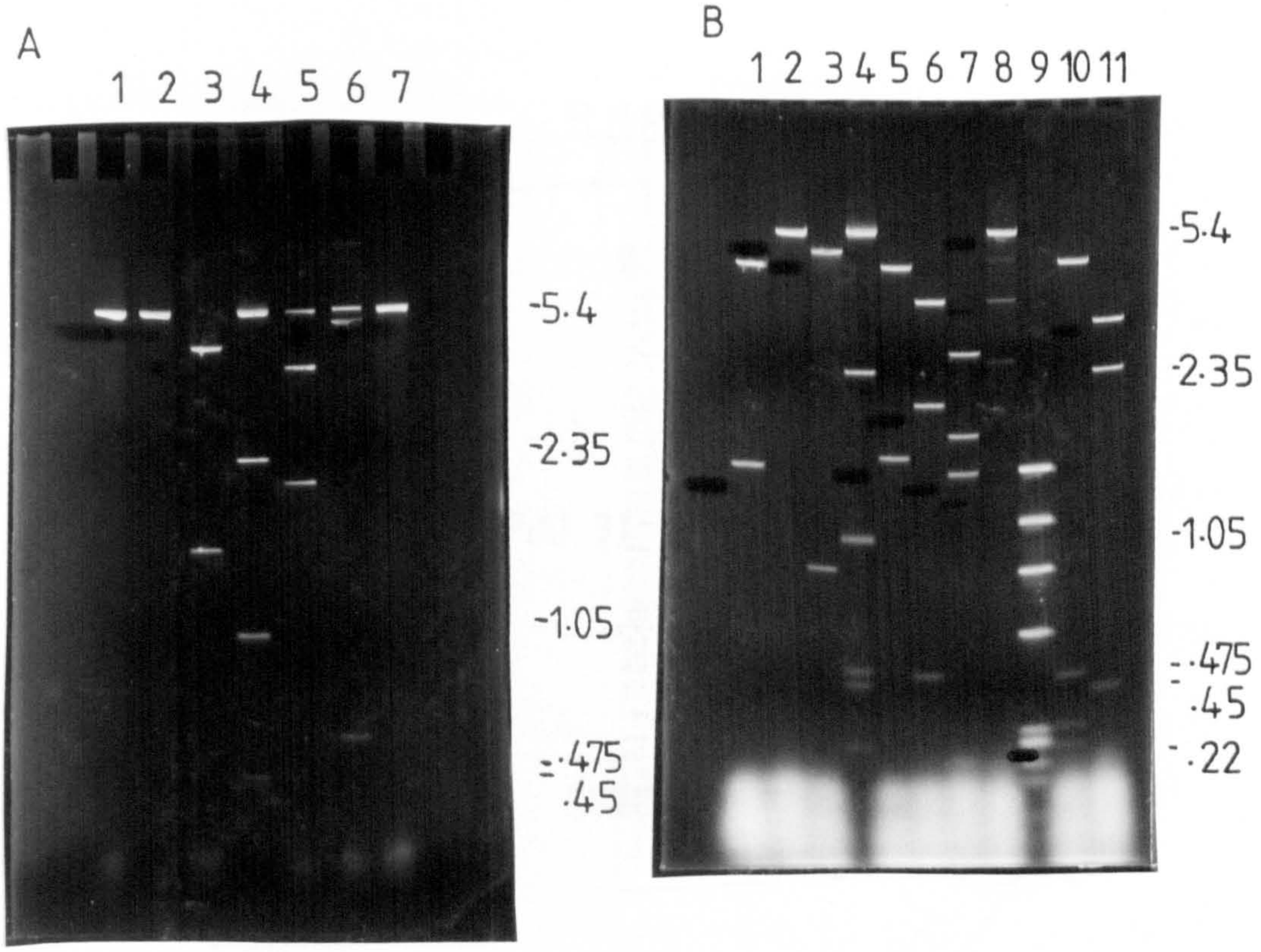


Figure 6.1 Restriction digests on pJJ71 and electrophoresis with Southern transfer of restricted DNA, hybridized with the 22 oligodeoxynucleotide. The autoradiograph of the Southern hybridization is shown in the overlay.

A. lane 1 Pst I; lane 2 EcoRI; lane 3 Sal I; lane 4 Marker, Hind III PM2; lane 5 Hind III; lane 6 Bam HI; lane 7 Pvu II.

B. lane 1 Pst I, Hpa I; lane 2 Pst I; lane 3 Pst I, Pvu II; lane 4 Marker, Hind III PM2; lane 5 Pst I, Sma I; lane 6 Pst I, Bgl II; lane 7 Bgl I; lane 8 Bal I; lane 9 Marker, Hae III øX 174; lane 10 Bst E II; lane 11 Ava I. Sizes indicated in base pairs x 10^{-3}.

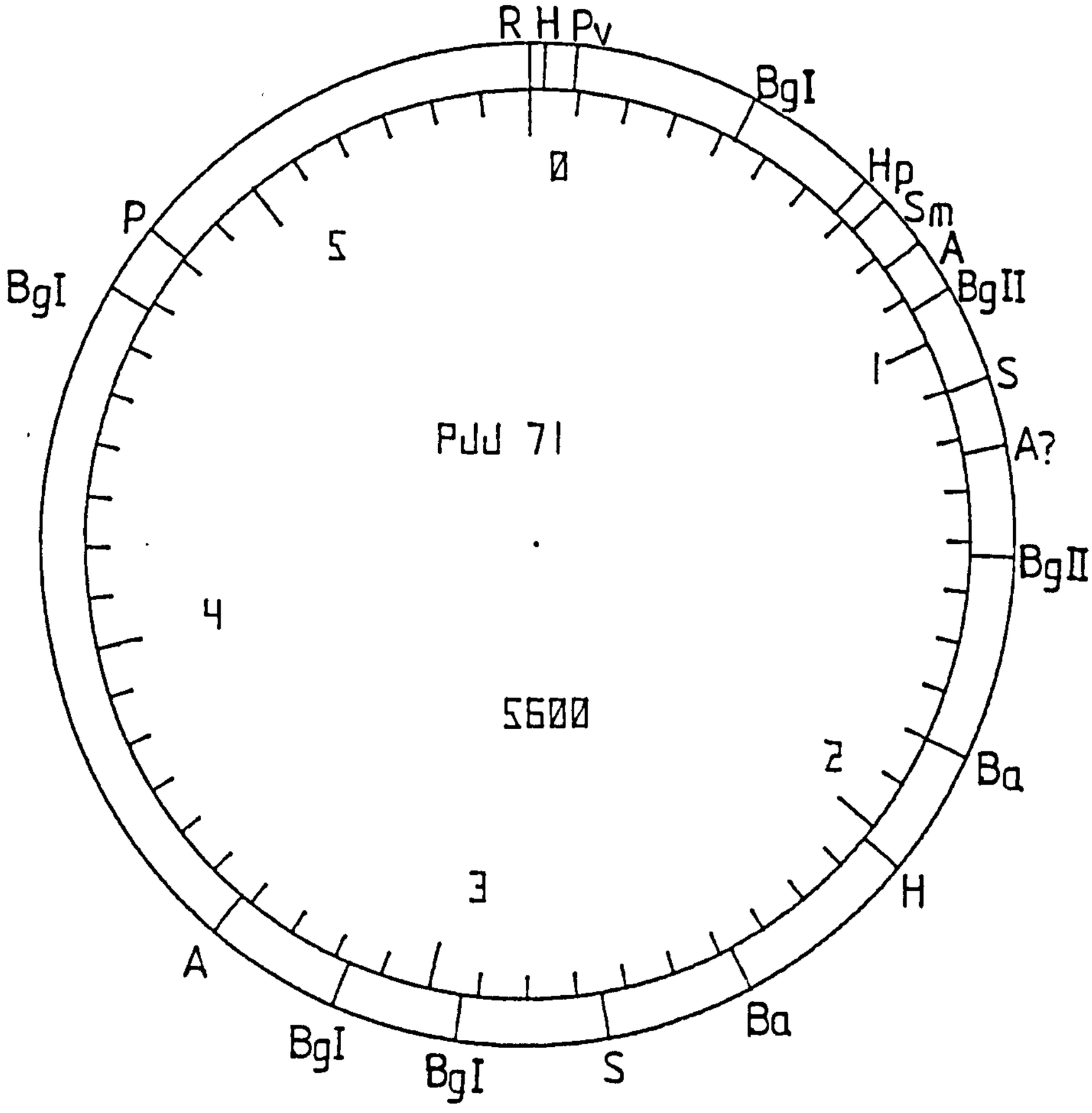


Figure 6.2 Restriction map of plasmid pJJ71 containing the ε gene cloned at the Hind III site of pAT153. Restriction sites: A, Ava I; Ba, Bam HI; BgI, Bgl I; Bg II, Bgl II; H, Hind III; Hp, Hpa I; P, Pst I; Pv, Pvu II; R, Eco RI; S, Sal I; Sm, Sma I. A? site uncertain.

the restriction fragments, as smaller fragments were not detected in these analyses. The main purpose of the basic restriction analysis was to determine sites useful for labelling prior to sequencing, the aim being to obtain the part of the amino acid sequence not completed by protein sequencing methods (Dorrington and Bennich, 1978) in the VH-D-JH-CH region of the Ig ε ND.

The sequencing strategy is shown in Figure 6-3 with sequencing in detail in the region of the VH-D-JH-CH junctions. The patterns of the sequencing reactions are shown in Figure 6-4. Some of the sequences obtained were unexpected because the 5' end of the Ig ε clone (relative to the mRNA) was inverted as judged by the amino acid data. This was interpreted as a cloning artifact as described by Volckaert et al., (1981)and observed by earlier workers (Chan et al., 1979). This is explained in Figure 6-5, where after hairpin and subsequent double strand formation, a double strand specific nick is introduced just after the priming site (on the other strand), which results in the hairpin unfolding with a portion of the site used to prime the second strand. This hairpin region is now inverted with respect to the rest of the double stranded region which enables it to prime the DNA polymerase I copying this region. As a result the region would be flanked by an inverted repeat, as found in the region of inversion. This result is not difficult to explain in the light of Volckaert et al., (1981) and others (Chan et al., 1979) results. Problems arise in the explanation of the presence of the 5' $(T)_{29}$ region, it is possible that this region was attached as a result of co-polymerization after the repair of the

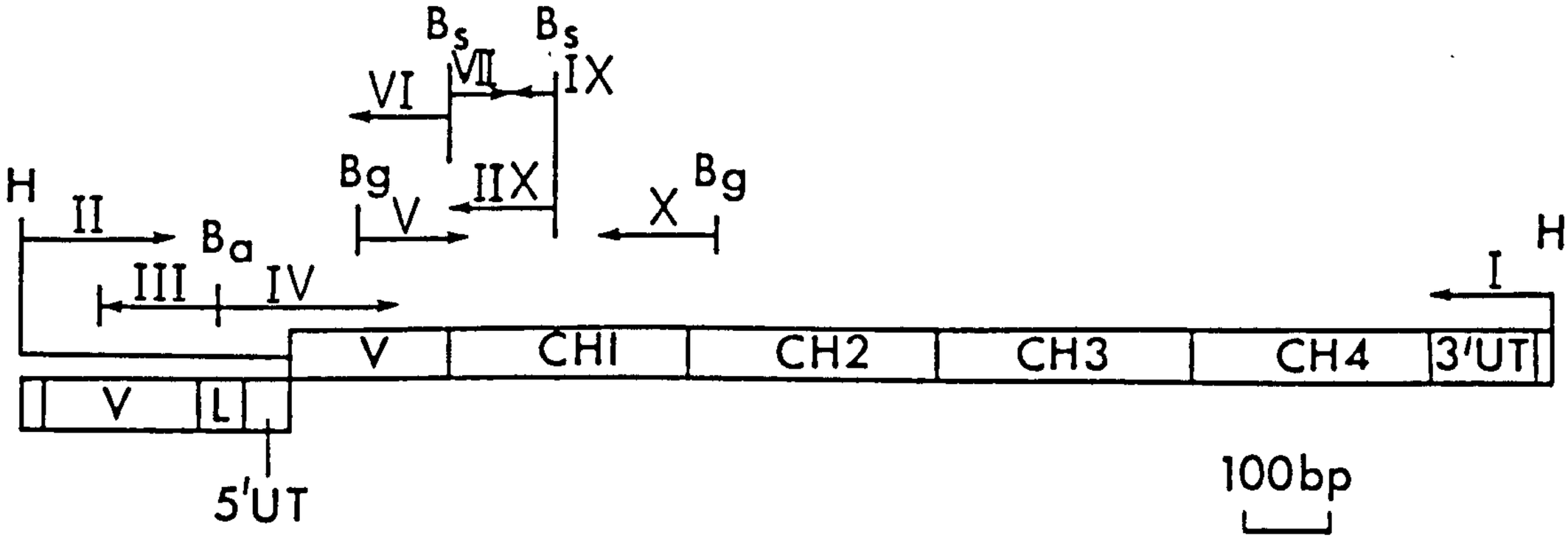
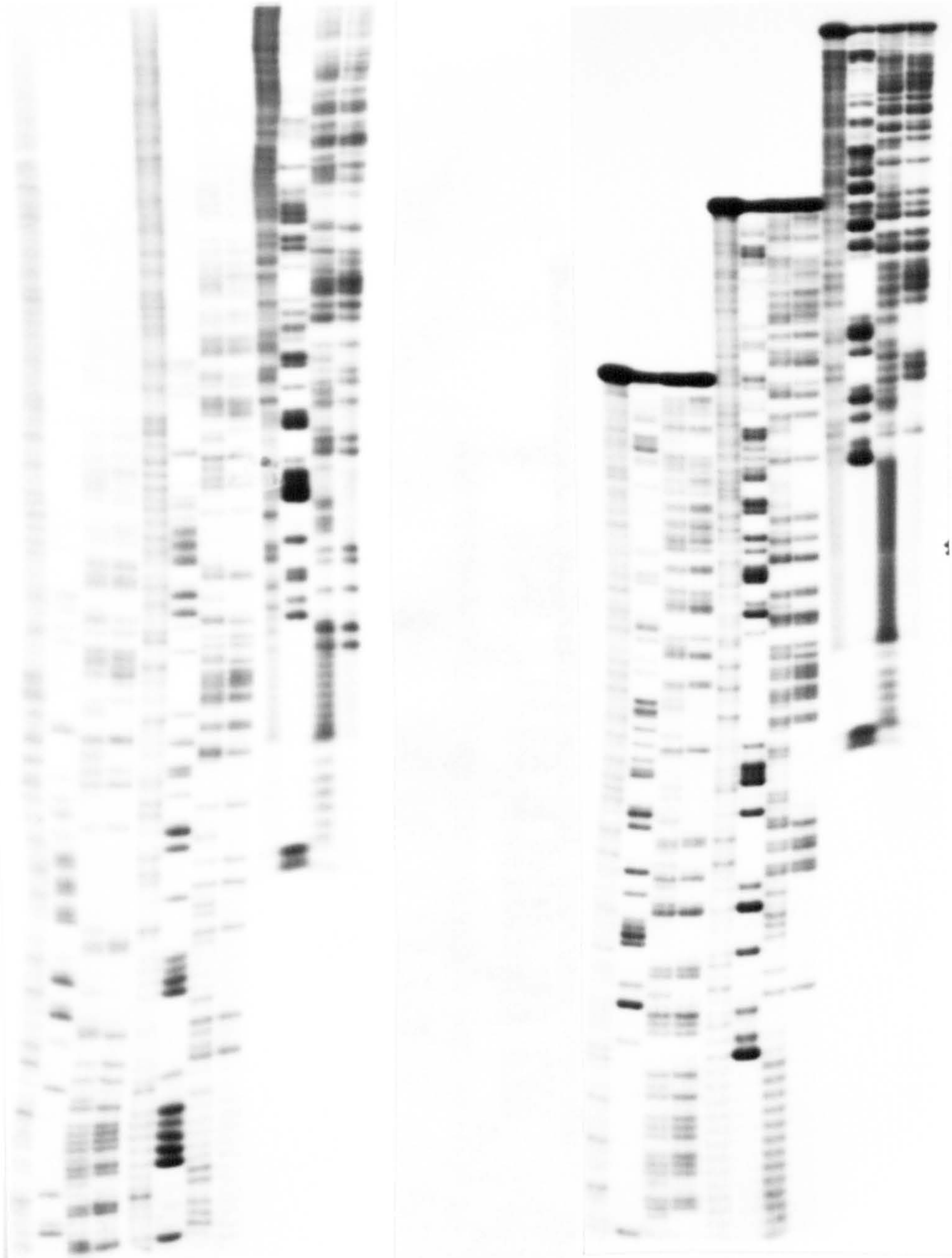


Figure 6.3 Sequencing strategy and restriction endonuclease sites used. H, Hind III; Ba, Bam HI; Bg, Bgl II; Bs, BstE II. The structure of the Ig ε clone sequence is also indicated.

I

II



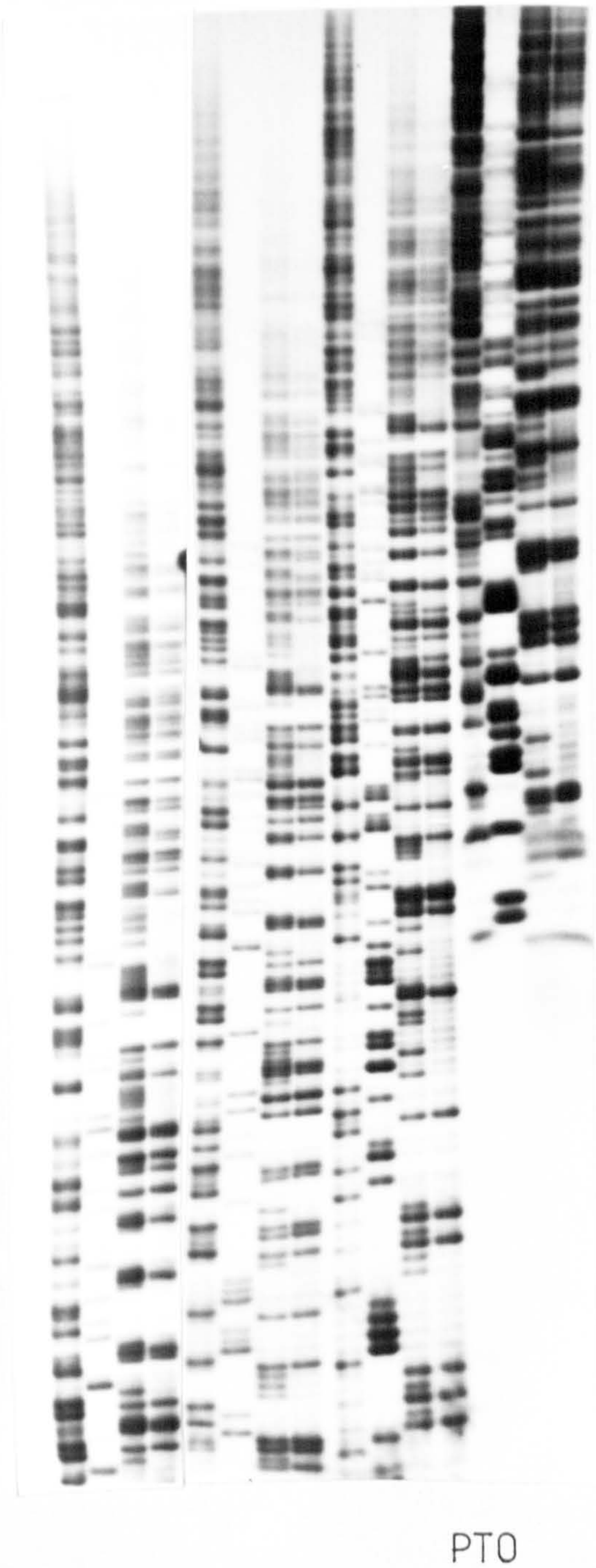
PTO

Figure 6.4 Autoradiograph of Maxam and Gilbert sequencing gels obtained from the Ig ε clone in pJJ71 gels I to XII as indicated in Figure 6.3. Sequencing reactions run on gels as A>C, G, TC then C.

III

PTO

IV



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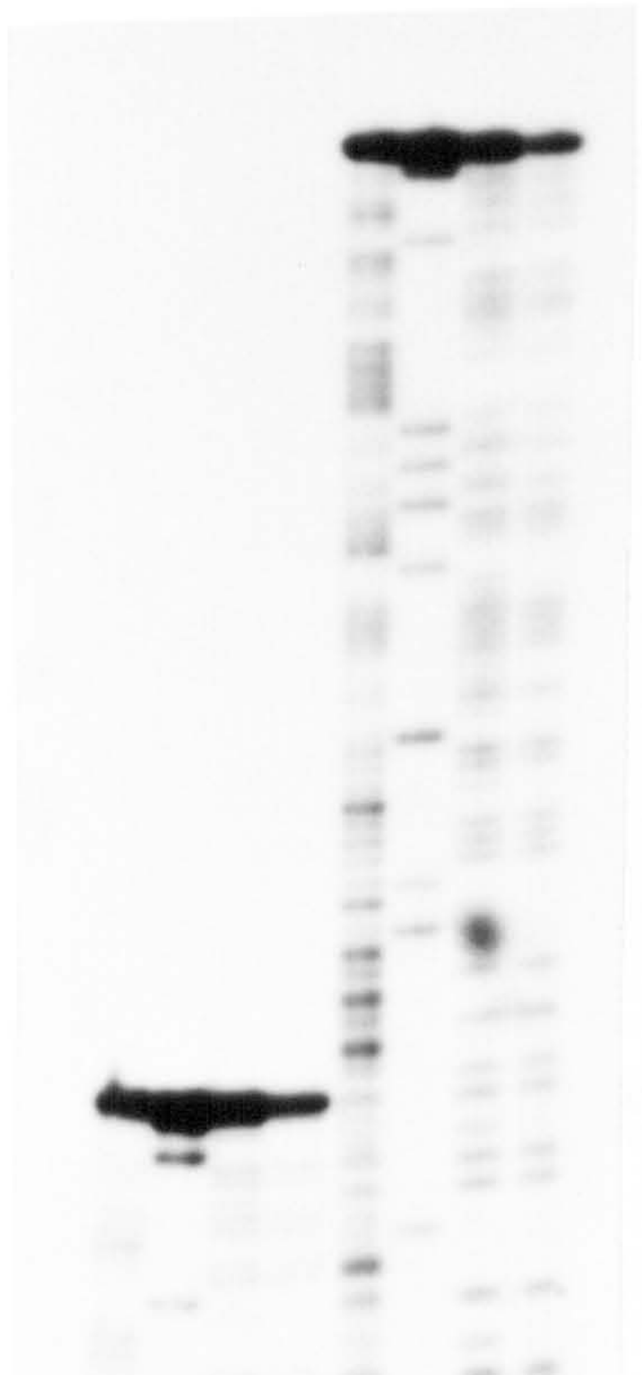
V

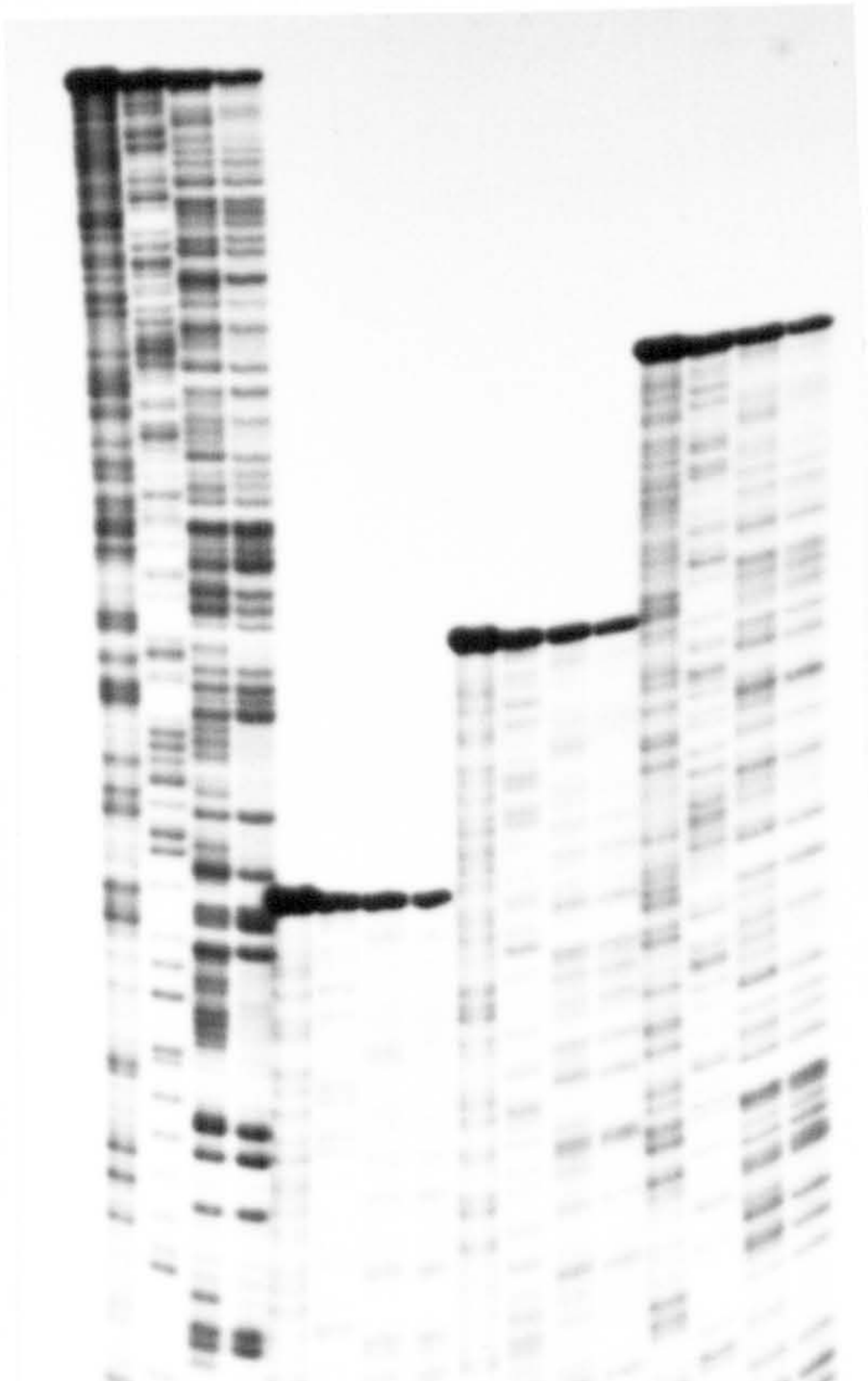
VI

PTO

VII

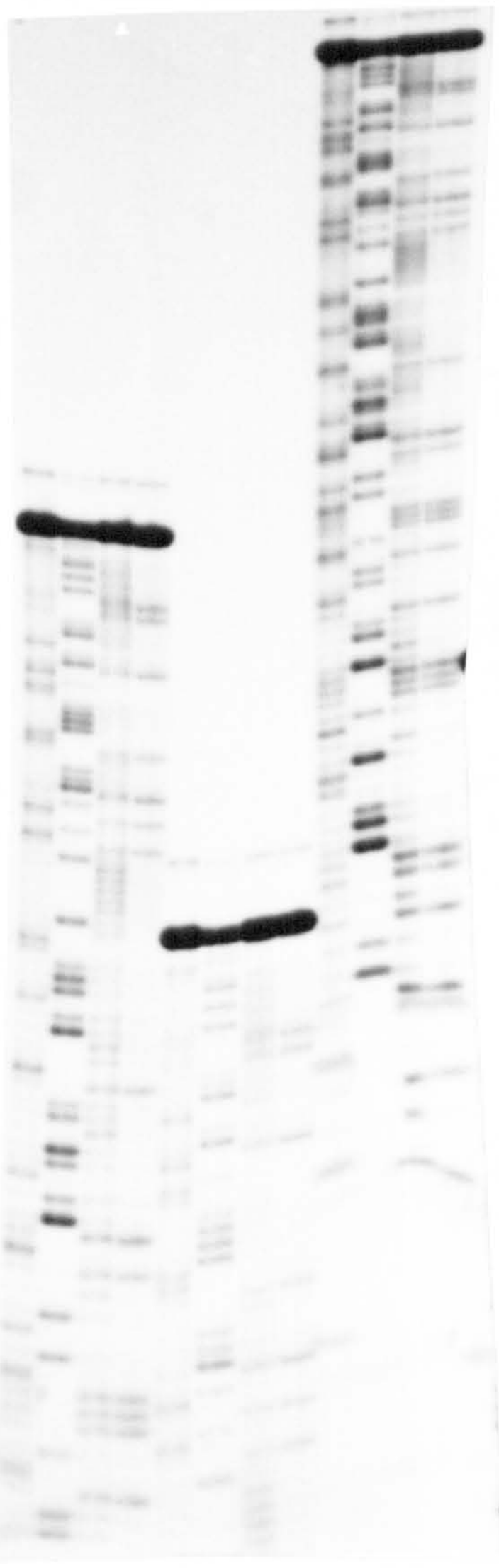


X

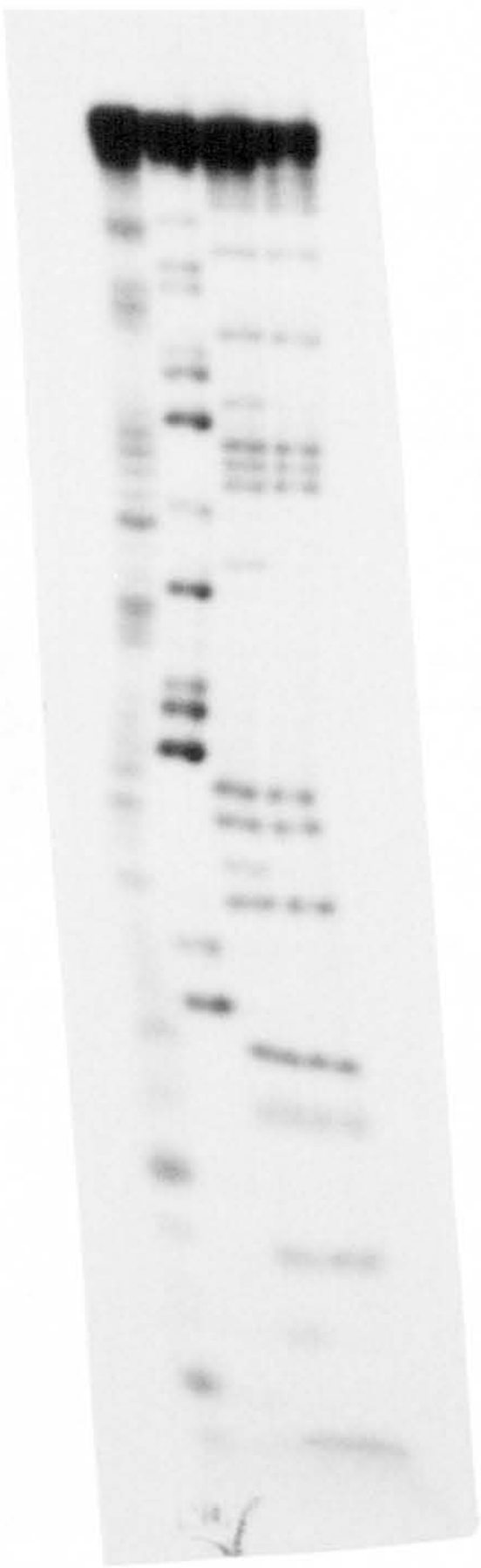


PTO

IIX



IX



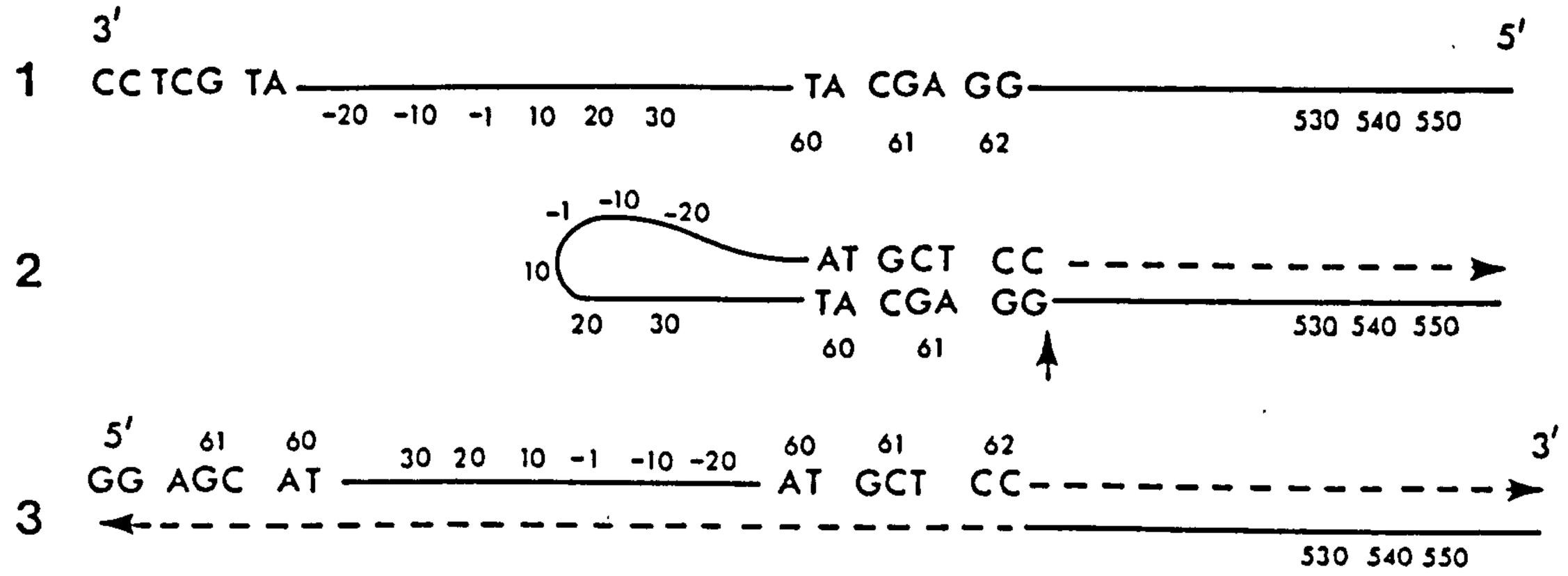


Figure 6.5 Postulated mechanism of formation of the Ig ε cloned sequence in pJJ71 during synthesis of the double-stranded cDNA : 1, reverse transcript of ε mRNA; 2, formation of a hair pin by complementary base pairing between the coding sequence for amino acid residues 60 - 62 and a sequence in the 5' untranslated region of the mRNA, synthesis of the second strand (dashed line), and nicking of the double stranded stem of the hair pin; 3, linearization of the molecule and extension of the cDNA (dashed line).

unfolded hairpin region with oligo dT as there was still the oligo dT primer from the Is cDNA synthesis present. Another possibility is that it was a polymer generated by the DNA polymerase I by slippage of a template, polymers of this type might be formed in low salt >50 mM or at high temperature, e.g. 30°C or higher (Adams et al., 1976, Wickens et al., 1978). This does seem unlikely as the conditions do not favour this and the last nucleotides before this region are two G,C base pairs which would not have provided a suitable template for this type of slippage polymerase extension, unless the DNA polymerase just added bases non-specifically. This is a possibility as DNA polymerase has been demonstrated to initiate polymer formation in the absence of a template, after a lag of the DNA polymerase starts incorporation into polymers of nucleotides (Adams et al., 1976).

The sequence at the site of the potential cloning artifact is shown (Figure 6-6). The possibility that this reflects a real mRNA sequence cannot be ruled out altogether. That it is real seems unlikely because of the absence of the necessary sequences to allow initiation and synthesis of a prepeptide for secretion. The production of secreted, processed ε heavy chain, has been shown for this cell line (Kenten et al., 1982). Also, the production by the 266BL cell line of functional fg E, having antibody binding sites characteristic of the original sequenced myeloma from which the cell line was derived (Nilsson et al., 1970) suggests that the sequence in this region of the clone is a cloning artefact.

The interpreted sequence for the ε gene sequence (Figure 6·7) shows some differences from the published amino acid sequence, this

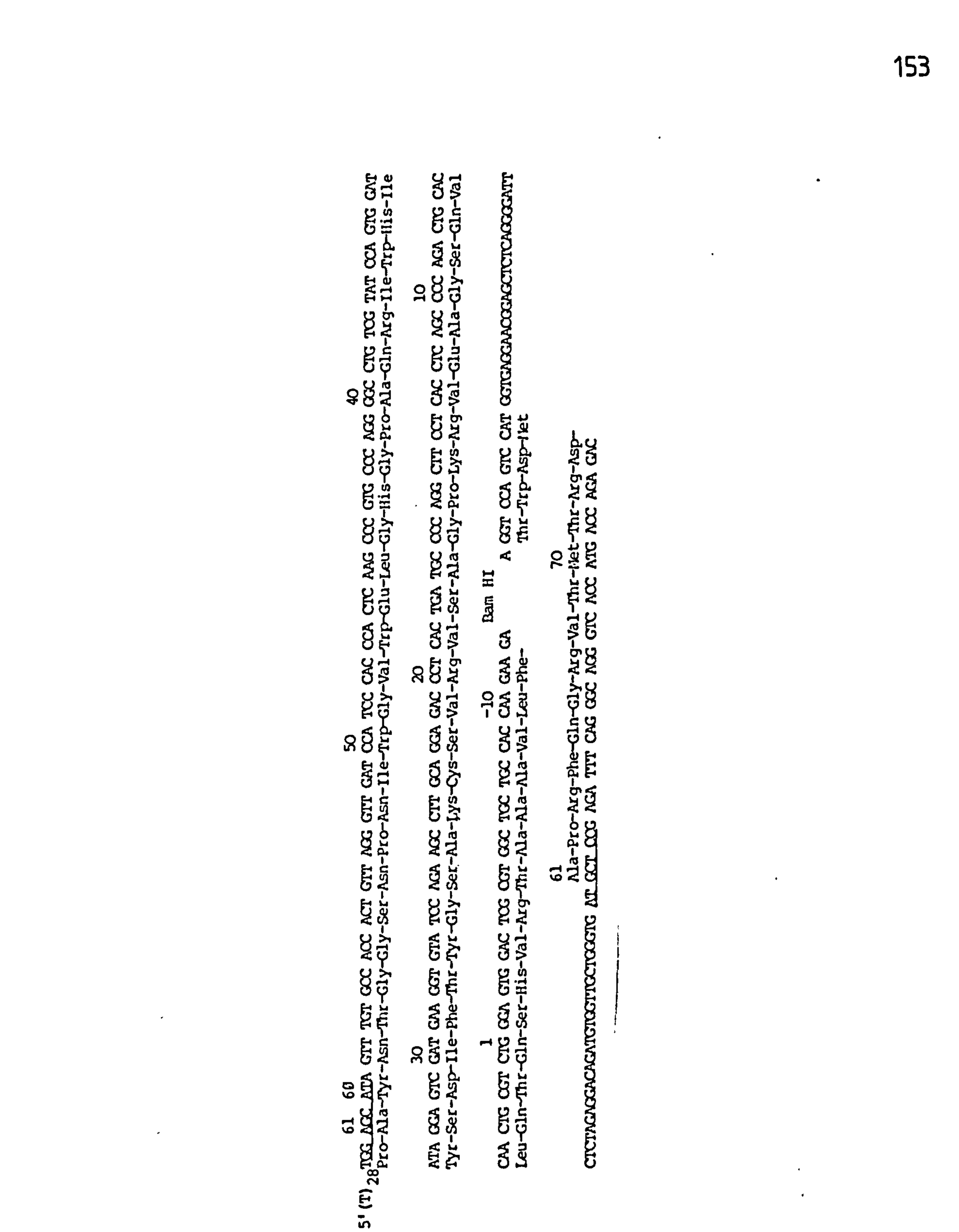


Figure 6.6 The cloned Ig ε sequence from pJJ71 at the site of the proposed cloning artifact showing (underlined) the inverted repeat of seven base pairs and the duplicated codons for amino acids 60 and 62, along with the remainder of the variable region sequence up to position 73. Amino acids set below nucleotide sequence are read from the complementary strand.

Figure 6.7 Hypothetical reconstruction of the ND ε chain cDNA : The DNA sequence and the determined amino acid sequence with insertions denoted by an asterisk (at positions 107, 221 and 222). Differences from Dorrington and Bennich (1978) amino acid sequence are noted above our sequence.

```
                                                                                                    Met-Asp-Trp-Thr
5'CACCCAGCAACCACATCTGTCCTCTAGAGAATCCCCTGAGAGCTCCGTTCCTCACC ATG GAC TGG ACC T                                        TC
                                                                                                                Bam HI
    -10                                    1  Val                                    10
Phe-Leu-Val-Ala-Ala-Ala-Thr-Arg-Val-His-Ser-Gln-Thr-Gln-Leu-Val-Gln-Ser-Gly-Ala-Glu-Val-Arg-Lys-Pro-Gly-Ala-Ser-Val-Arg
TTC TTG GTG GCA GCA GCC ACG CGA GTC CAC TCC CAG ACG CAG TTG GTG CAG TCT GGG GCT GAG GTG AGG AAG CCT GGG GCA TCA GTG AGG

20                                    30                Val Gly                 40                                    Ile His
Val-Ser-Cys-Lys-Ala-Ser-Gly-Tyr-Thr-Phe-Ile-Asp-Ser-Tyr-Ile-His-Trp-Ile-Arg-Gln-Ala-Pro-Gly-His-Gly-Leu-Glu-Trp-Val-Gly-
GTC TCC TGC AAG GCT TCT GGA TAC ACC TTC ATC GAC TCC TAT ATC CAC TGG ATA CGA CAG GCC CCT GGG CAC GGG CTT GAG TGG GTG GGA

50                                    60                                    70
Trp-Ile-Asn-Pro-Asn-Ser-Gly-Gly-Thr-Asn-Tyr-Ala-Pro-Arg-Phe-Gln-Gly-Arg-Val-Thr-Met-Thr-Arg-Asp-Ala-Ser-Phe-Ser-Thr-Ala-
TGG ATC AAC CCT AAC AGT GGT GGC ACA AAC TAT GCT CCG AGA TTT CAG GGC AGG GTC ACC ATG ACC AGA GAC GCG TCC TTC AGT ACA GCC

80                                    90                                    100                                   *
Tyr-Met-Asp-Leu-Arg-Ser-Leu-Arg-Ser-Asp-Asp-Ser-Ala-Val-Phe-Tyr-Cys-Ala-Lys-Ser-Asp-Pro-Phe-Trp-Ser-Asp-Tyr-Tyr-Asn-Phe-
TAC ATG GAC CTG AGA AGT CTG AGA TCT GAC GAC TCG GCC GTG TTT TAC TGT GCG AAA AGT GAC CCT TTT TGG AGT GAT TAT TAT AAC TTT

110                                   120                                   130
Asp-Tyr-Ser-Tyr-Thr-Leu-Asp-Val-Trp-Gly-Gln-Gly-Thr-Thr-Val-Thr-Val-Ser-Ser-Ala-Ser-Thr-Gln-Ser-Pro-Ser-Val-Phe-Pro-Leu-
GAC TAC TCG TAC ACT TTG GAC GTC TGG GGC CAA GGG ACC ACG GTC ACC GTC TCC TCA GCC TCC ACA CAG AGC CCA TCC GTC TTC CCC TTG

140                                   150                                   160
Thr-Arg-Cys-Cys-Lys-Asn-Ile-Pro-Ser-Asn-Ala-Thr-Ser-Val-Thr-Leu-Gly-Cys-Leu-Ala-Thr-Gly-Tyr-Phe-Pro-Glu-Pro-Val-Met-
ACC CGC TGC TGC AAA AAC ATT CCC TCC AAT GCC ACC TCC GTG ACT CTG GGC TGC CTG GCC ACG GGC TAC TTC CCG GAG CCG GTG ATG
                                                                                                                Bst Ell

                                      200                                   210
                Thr-Ile-Ser-Leu-Leu-Thr-Val-Ser-Gly-Ala-Trp-Ala-Lys-Gln-Met-Phe-Thr-Cys-Arg-Val-Ala-His-Thr-Pro-Ser-Ser-
                ACC ATC AGC TTG CTG ACC GTC TCG GGT GCG TGG GCC AAG CAG ATG TTC ACC TGC CGT GTG GCA CAC ACT CCA TCG TCC

220 *  *                              230                                   240
Thr-Asp-Trp-Val-Asp-Asn-Lys-Thr-Phe-Ser-Val-Cys-Ser-Arg-Asp-Phe-Thr-Pro-Pro-Thr-Val          Gly-Lys Ter
ACA GAC TGG GTC GAC AAC AAA ACC TTC AGC GTC TGC TCC AGG GAC TTC ACC CCG CCC ACC GTG          GGT AAA TGACGTACTCCTGCCT
                                                                                 Bgl II

                                                                    Ter
CCCTCCCTCCCATGGCTCCATCCAGCTGTGCAGTGGGGAGGACTGGCCAGACCTTCTGTCCACTGTTGCAATGACCCCAGGAAGCTACCCCCAATAAACTGTGCCTGCTCAGAGAA(A)12 .
```

completes the amino acid sequence for the Ig E ND, ε heavy chain. The amino acid differences in the constant region, were also demonstrated by the germline genomic sequence of Leder's group (Max et al., 1982) indicating that they were not due to polymorphism as these authors suggested. These workers also showed other discrepancies which indicated that the published amino acid sequence could not be relied on totally, or, that there was polymorphism. This small variation from the amino acid sequence was also demonstrated on publication of the Ig E ND ε genomic sequence which agreed with the germline sequence with the exception of one amino acid Trp in the CH_4 domain, which in germline sequence appears as a Ile, (Flanagan and Rabbitts, 1982).This may be a polymorphism or a sequencing error as the germline gene is only sequenced on one strand at this point. The small variation in the variable region sequence did not appear to be due to mutational events as the amino acid differences at positions 34,35 and 48,49 indicated that peptides had been placed incorrectly in the protein sequence as the differences to our sequence at 34,35 is found at 48,49 and vice versa and the two amino acids COOH to their discrepancies are Trp-Ile. This may have led to the confusion over their position. In the case of the residue at position 2, the presence of Thr in place of the published Val would require two nucleotide changes ACG→GTG. This seems unlikely because the remaining variable region sequence does not exhibit any evidence of single nucleotide changes within codons indicating a further error.

The comparison of this variable region nucleotide sequence of subclass I with that for variable region subclass III (VH III)

(Matthyssens and Rabbitts, 1980) indicate a large degree of homology (58%, Figure 6-8). This homology is supported by the use of a V_H III closely related to the one in Figure 6-8 in hybridization to the Ig E ND ε genomic variable region (Flanagan and Rabbitts, 1982). The lowest degree of homology is found in the 5' untranslated region, and the hyper-variable regions, as would be expected. The degree of homology is also low in the region of the prepeptide, possible due to a comparison of a differing variable region subclass, rather than evidence for diversity in this region. This possibility also exists for the other sequences compared.

The human VH III (Matthyssens and Rabbitts, 1980) and human JH region (Ravetch et al., 1981) sequences has enabled the prediction of these regions in the Ig ε ND sequence. The D region in Ig ε ND is thought to cover amino acids 99-115 and has been compared for homology with previously published D regions mainly from man (Ravetch et al., 1981; Siebenlist et al., 1981) but also some from mouse (Kurosawa and Tonegawa, 1982). These results are shown in Figure 6-9. They show the presence of homology similar to that indicated by Ravetch et al., (1981) for the 5' half of 'D35' with the 3' half of 'D36'.The presence of vestiges of internal recombination signals is an interesting feature. These could have constituted a redundant signal within the Ig ε ND D region. The presence of internal functional recombination signals has been proposed (Kurosawa and Tonegawa, 1982). The homology demonstrated here probably does not provide alternative splicing signals as it lies. outside the consensus sequences for these signals (Sakano et al., 1981).

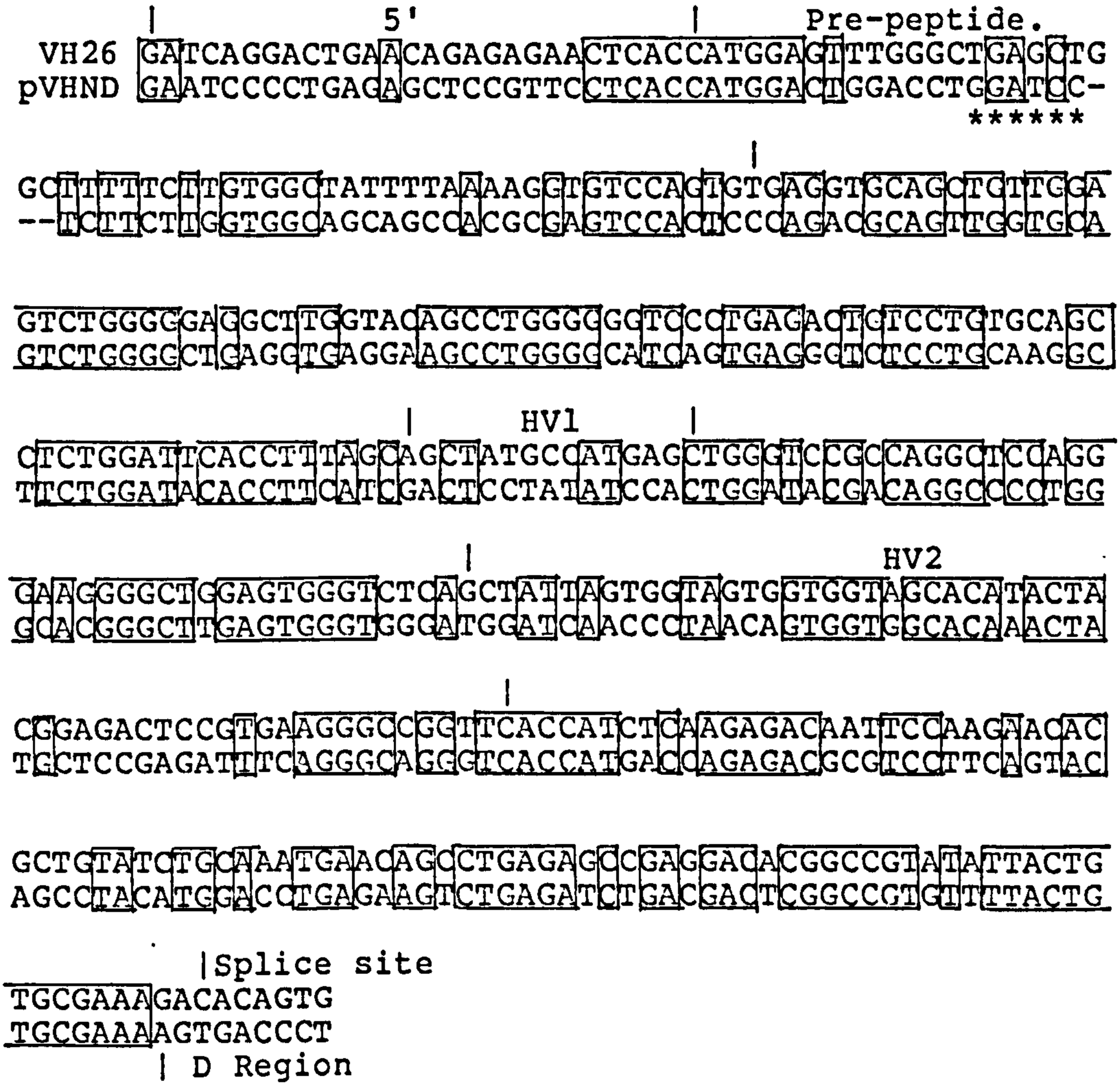


Figure 6.8 Comparison of human variable region nucleotide sequence, VH26, variable region subgroup III genomic sequence (with introns removed, Matthyssens and Rabbitts 1980) with VH ND, variable region subgroup I. Homologies are boxed, Bam HI site (*) inserted on the basis of homology. Positions of HV1 and HV2 as Early et al (1980). Total homology = 58%.

```
VH          |                                'D region'                                                    | JH6
ND epsilon   Ser Asp Pro Phe Trp Ser Asp Tyr Tyr      Asn Phe Asp  Tyr Ser   Tyr Thr Leu
             AGT GAC CCT TTT TGG AGT GAT TAT TAT --- AAC TTT G-AC TAC TC--G TAC ACT TTG

D35    GGATC CTG GGG CCG TAT AGC AGT GGC TGG TAC CCG AAC TCG G-AC

D36                                              AG --- GAT ATT GTAC TAA TGGTG TAT GCA TTG GCCTT

DHQ52                                            CT --- AAC TGG GGA

FL16                                                    TT T-AT TAC TACGG TAG TAG CTA C

SP2  family                                 TC TAG TAT GGT AAC TAC
                                            C   C     A   T   G

                                            TC TAG TAT --- GAT TAC G-AC
                                            C   C           G   A   T

                                            TC TAC --- TAT GGT T-AC TAC
                                            C   G           A   A   G

D 'genomic'      GGT TTT TGG< 12 BASES > --- CAC TGT G<  D region  >C ACT GTG<12BASES>
```

Figure 6.9 Comparison of Ig ε ND 'D region' nucleotide sequence with D regions from man D35, D36 and DHQ 52 (Ravetch et al 1981) and D regions frommouse FL16 and SP2 family (Kurosawa and Tonegawa 1982). A comparison is also shown for D region splice sequences. Homology is underlined.

The JH region used in the ND ε heavy chain was initially identified as JH4 from homology with mouse JH sequences. With publication of the human JH germline locus, however its identification as JH6 was possible (Ravetch et al., 1981). This would explain the weak signal found for JH region probes to the genomic DNA of the cell line 266BL, with this J region being at the end of the JH gene locus (Flanagan and Rabbitts, 1982). This JH region does contain a base which differs from the germline JH6, a G to C conversion, which may represent a somatic mutation, as demonstrated in mouse (Givol et al., 1981). The JH region in the ND Ig ε heavy chain has been truncated by 21 base pairs on recombination with the D segment. This type of event has been demonstrated for mouse JH region (Sakano et al., 1981). The 3' terminal sequence of the ND Ig ε gene demonstrates the presence of the sequence 5' GGTAAA 3' which may constitute a site for the splicing of the message precursor into a down stream sequence to encode an elongated form of the ε chain which is bound to the cell membrane rather than secreted. (Rogers et al., 1981).

Analysis of 266BL mRNA (8,34)

The poly A^+ RNA from the cell line 266BL was analysed by gel electrophoresis after glyoxal treatment in order to denature it. The RNA was transferred to nitrocellulose paper and hybridized with nick translated pJJ71 (see Methods). The result (Figure 6-10) giving a size for the mRNA of 2150 ± 100 bases, this is in agreement with values for rat (Zajdel-Blair et al.; 1981, Hellman et al., 1982; Faust and Moore, 1981) and mouse Ig ε mRNAs (Ishida et al., 1982). The presence of 3 distinct bands above the major hybridizing

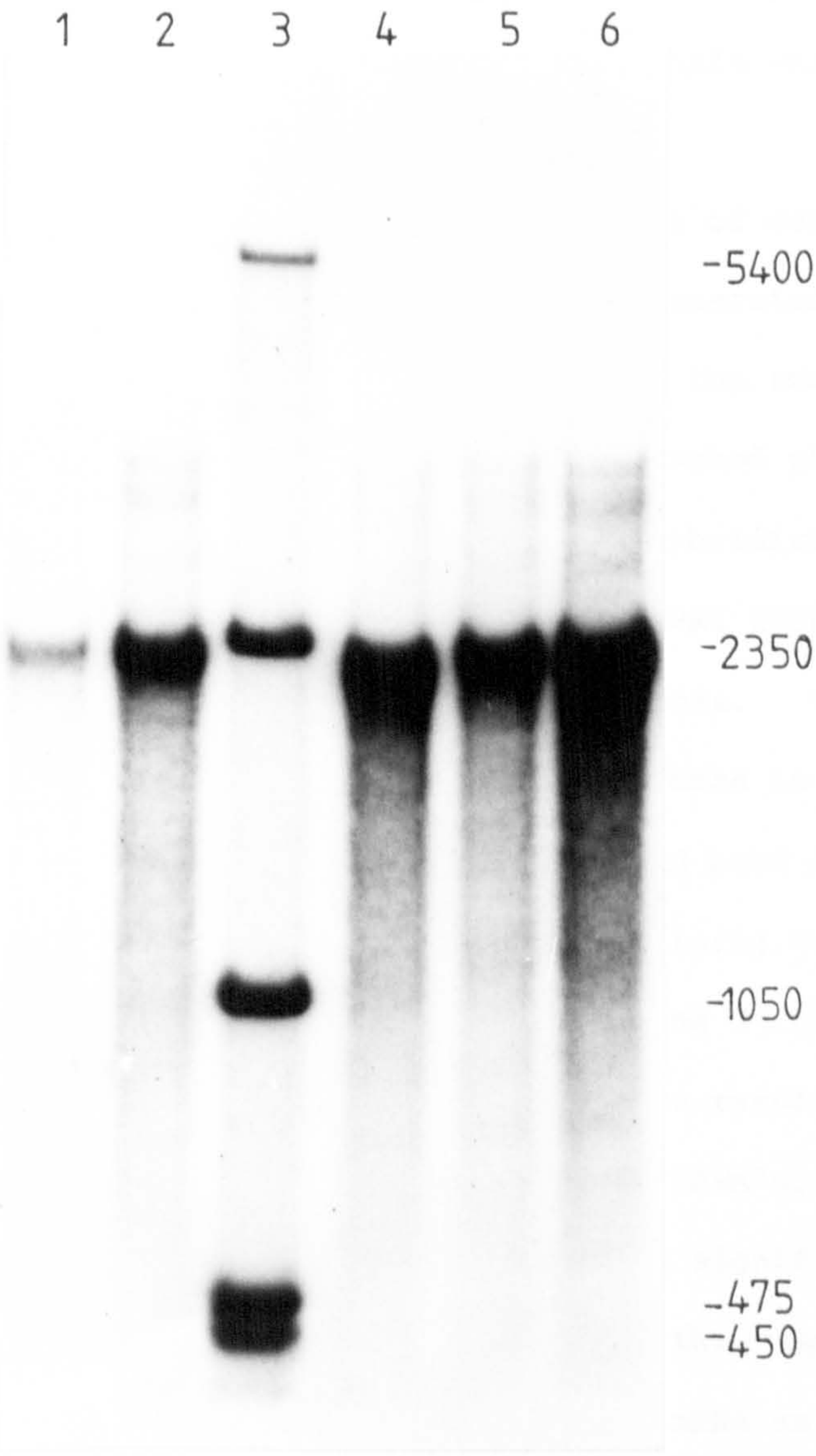


Figure 6.10 Electrophoresis and autoradiography of 266 BL poly A+ RNA analysed by glyoxal gel electrophoresis and hybridized with [^{32}P] labeled pJJ71. Lanes 1, 2, 4, 5 and 6; 5 µg of differing 266 BL poly A+ RNA preparations, lane 3 [^{32}P] labeled Hind III digest of PM2 DNA. Not shown, 5 µg of RPMI 1788 poly A+ RNA (gift from Dr. J.M.B. Warwick) gave no detectable hybridization on this exposure. Sizes indicated in bases.

RNA species indicated the presence of precursor RNA species and or mRNA specifying the elongated form of the Ig ε chain which is bound to the cell membrane rather than secreted.

In order to test this possibility a fragment of our cloned ε gene containing the 3' untranslated region was generated by a Pvu II and Bam HI digest of PJJ71, the largest of the two fragments being gel purified. The fragment, with its attached pAT153 sequence to improve the signal, was nick translated and hybridized to glyoxal gel electrophoresed poly A^+ RNA from 266BL and RPMI 1788 (gift from Dr. J. M. B. Warwick) transferred to nitrocellulose. This gave the result shown in Figure 6-11, indicating no difference in the profile of hybridizing bands. If one of these bands had been derived from the membrane bound Ig ε heavy chain, the RNA splicing events to add the membrane binding signal would have removed the 3' region found for the secreted Ig ε heavy chain and used as a probe, resulting in loss of hybridization to the higher molecular weight mRNA's. If the 3' region of the Ig ε membrane bound form contained significant homology to the 3' region of the Ig ε secreted form, then this approach would not have detected these higher molecular weight mRNA as Ig ε membrane bound forms. This would indicate they are precursors.

Prolonged exposure of this filter led not only to the development of two bands in the RPMI 1788 (Ig μ secreted and membrane bound producing cell line Molgaard et al., (1981)) track but also to the general background on the track. It is possible these represent transcripts of the ε gene locus, as would be expected in the lympho-cytes described by Yaoita et al., (1982), these also expressing

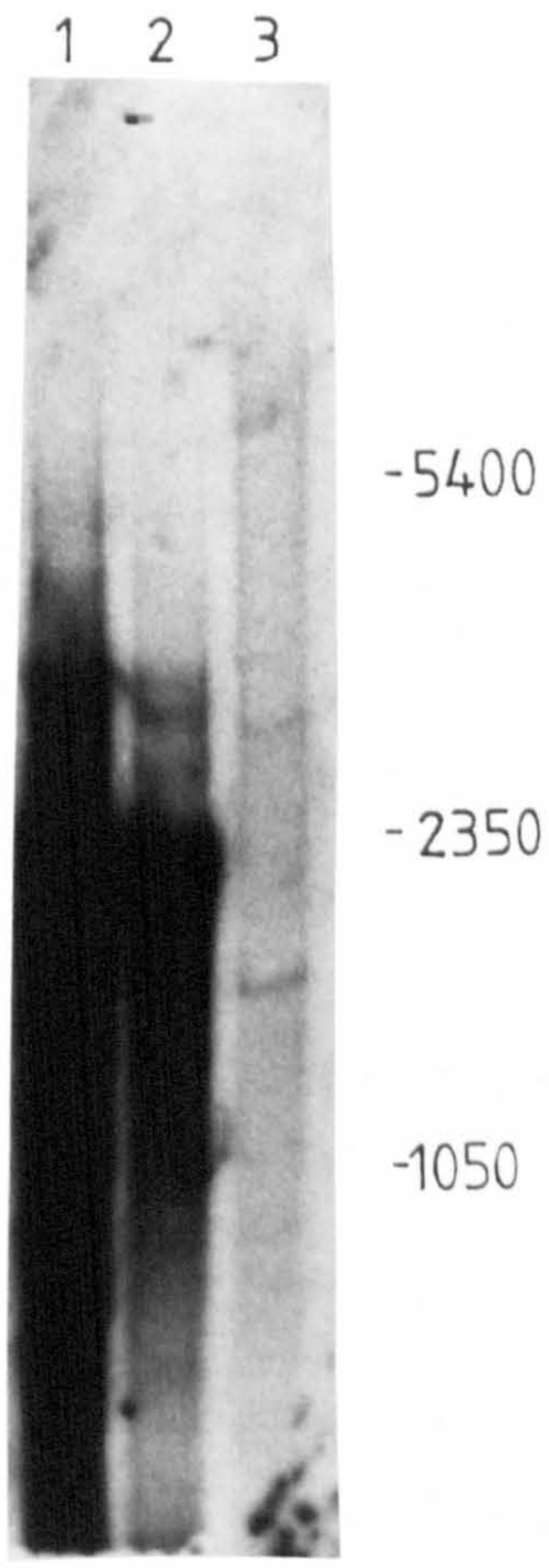


Figure 6.11 Electrophoresis and autoradiography of 266 BL and RPMI 1788 poly A+ RNA, analysed by glyoxal gel electrophoresis. Hybridization with [^{32}P] labeled 3' untranslated region of the Ig ε clone from plasmid pJJ71 as a Pvu II to Bam HI fragment. The fragment also contains 3.2Kb of pAT153. 266 BL poly A+ RNA (lanes 1 and 2) and RPMI 1788 (lane 3). Sizes indicated in bases.

surface Ig M, but more likely represent some homology with the 3' region of the ε gene, or nonspecific binding as the intensity is very low relative to the result with 266BL mRNA.

CHAPTER 7

Expression of Immunoglobulin epsilon clone in E. coli

The expression of eukaryotic genes in prokaryotic systems has been the subject of much interest and many genes of eukaryotic origin have been expressed. In this study we were interested in expression of part of the Ig ε heavy chain expected to contain the amino acids necessary for the formation of the (Fc) structure required for binding to Ig E receptors present on mast cells. The bacterial expression of an Ig ε fragment with activity would ultimately allow further refinement of ideas regarding the structures involved in the binding to mast cells. Binding of Ig E to mast cells via receptors is ultimately responsible for the clinical manifestations of allergy, see Introduction.

The construction of an expression clone containing an appropriate gene segment was achieved by using the expression plasmids described by Tacon et al., (1980). These 3 plasmids allow expression of chimeric proteins, each plasmid in a differing reading frame. The plasmids contain from 9 to 14 amino acids at the NH_2 terminus, made up from 7 amino acids of the E. coli Trp E gene product, the remainder from Hind III linkers inserted for the phase change and for a cloning site. The expressed chimeria is under control of the tryptophan promoter/operator including the attenuation region (as described in the Introduction).

Two possible gene fragments could be isolated from the clone pJJ71 and used, either a Bgl II to Hind III or a Sal I to Hind III fragment, coding for the domains of interest which are found in the Fc region,

namely the CH_2, CH_3 and CH_4 domains.

The Bgl II - Hind III fragment would have the NH_2 terminus of ε protein starting 5 amino acids from one of the CYS's involved in the inter-chain disulphide bridges between two Ig ε heavy chains forming the functional Fc region. This proximity of the inter-chain CYS to the fusion with TrpE and linker derived amino acids of the expression vector was considered to be undesirable as these sequences might interfere in the normal disulphide bond formation.

The Sal I - Hind III fragment has the NH_2 terminus of the ε protein at amino acid 222 (Dorrington and Bennich, 1978). This would include a sequence from the CH1 domain (The COOH terminal β strand of CH1, by homology with Ig G γ chain domain (Polijak, 1978) and also from predictive secondary structures by a number of methods performed at Leeds University for Dr. J. Pardon), with the Ig ε gene starting 7 amino acids from the COOH terminal CYS of the CH1 domain. This domain is unusual in having two intra-domain disulphide bridges, with the extra disulphide bridge thought to be between the NH_2 terminal and COOH terminal β strands of the domain as found for some Ig G γ chains (Dorrington and Bennich, 1978).

The cloning of the Sal I - Hind III fragment was chosen as it contained more Ig ε sequence NH_2 terminal to the inter-chain CYS than the Bgl II - Hind III fragment, it was hoped this would be more favourable to the formation of the inter-chain disulphide bridges. The expression vector felt to be best suited was one producing a fusion with the shortest number of extra amino acids to the NH_2 termini of the Ig ε gene product. The vector pWT211 having only 9 amino acids derived from the Trp E and linker regions was chosen.

In order to clone the Sal I - Hind III fragment it was decided to regenerate a Hind III site by cloning in to pAT153 rather than either clone directly into the expression vector or by addition of linkers. The cloning to regenerate the Hind III site was into pAT153's Hind III site, pAT153 was chosen as the fragment should not be expressed, which might interfere with the cloning of the gene, if the expressed product were toxic to E. coli this would reduce the cloning efficiency and produce only inserts in the incorrect orientation. This strategy would enable production of the Hind III fragment for a subsequent cloning into pWT211 and transformation of WT217. The strategy is outlined in Figure 7-1, showing the reconstruction of the Hind III site to provide the phasing of the gene fragment for expression in pWT211. The expression plasmid containing the Ig ε gene was used to transform WT217. The transformants were then screened for inserts and their orientation was determined using plasmid DNA prepared by the quick boiling lysis method followed by restriction digestion with Pst I and Bgl II and analysis of the DNA fragments by gel electrophoresis (27,18).

A clone designated pSC213 containing the inserted gene in the correct orientation was further investigated by studying the control of the expression expected for genes inserted under control of the tryptophan operon. The plasmid was also then transformed into HB101 (28).

The bacteria containing the expression plasmid with and without the Ig ε gene insert were grown in the presence and absence of tryptophan (100 μg/mL), repressed and derepressed respectively, and with induction

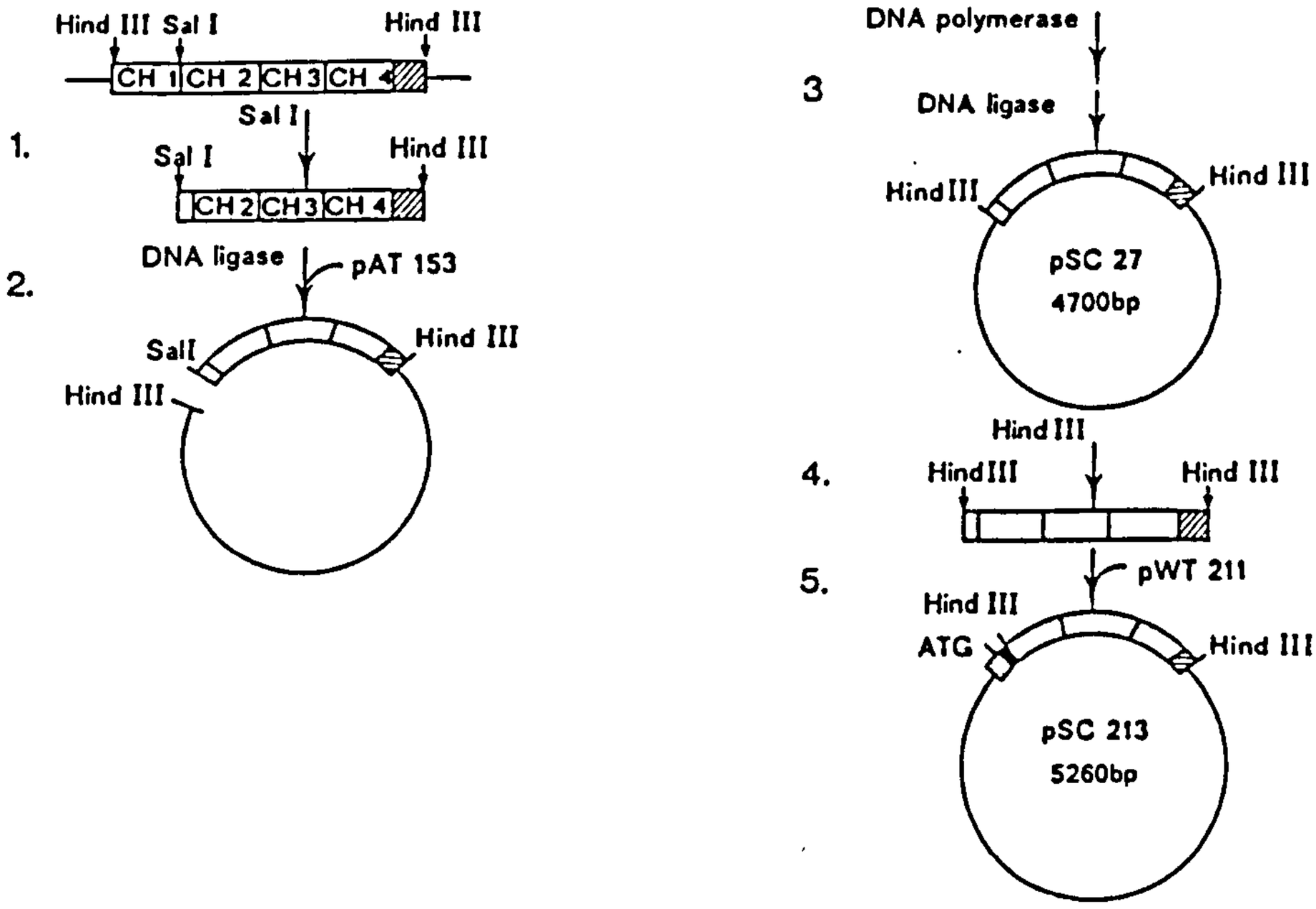


Figure 7.1 Construction of Plasmids for Controlled Bacterial Synthesis of a Human ε Chain Fragment.

The following steps of the plasmid construction are illustrated:

(1) isolation of the 1,140 bp SalI-HindIII fragment from pJJ71;

(2) ligation of the fragment to HindIII cut and E.coli bacterial alkaline phosphatase treated pAT153;

(3) filling in the recessed ends of the recombinant DNA with DNA polymerase I and blunt end ligation to regenerate a HindIII site in pSC27;

(4) after transformation of E.coli MRC8 and isolation of the plasmid, recovery of the HindIII inserted sequence;

(5) ligation of the recovered sequence to HindIII cut and E.coli bacterial alkaline phosphatase treated pWT211, followed by transformation of E.coli WT217, to give controlled expression of the immunoglobulin gene fragment.

using 3-β-indole acrylic acid (10 μg/mL) in M9 medium supplemented as described (24). The results of this experiment are shown in Figure 7-2 with samples being taken at A_{600nm} of ∿0.5 and ∿1.0. The growth of bacterial cultures of WT217 containg pSC213 on induction was markedly slower than any of the other cultures.

Protein from the bacterial pellet was obtained by solubising the whole pellet in Laemmli (1970) gel loading buffer supplemented with 6M urea, 2 mM PMSF, 5% (v/v) 2-mercaptoethanol and 10 mM EDTA, followed by heating at 100°C for 5 minutes and analysis by gel electrophoresis (10). Typically the pellets from 1 mL of culture were taken up in 40-80 μL of the above modified loading buffer and analysed on a 10% acrylamide gel, followed by transfer to a nitrocellulose filter, stained with amido black and probed with (^{125}I) labelled affinity purified rabbit anti Ig E specific to the CH_3 and CH_4 domains (gift from Dr. Ishizaka) (12). The results of the immunodetection (Figure 7-3) demonstrate the production of Ig ε antigen under control of the tryptophan operon, as expected. Production of this polypeptide correlated with the production of a significant amount of total bacterial protein of the same molecular weight, as judged by the stained protein transfer (Figure 7-2). The level of the stained polypeptide and the antigenic activity appear to be higher in the sample taken at an absorbance of 1.0 than that taken at 0.54, indicating some accumulation of product. The molecular weight of the expressed protein was expected to be 37,500 daltons, determined from amino acid composition (Max et al., 1982, Tacon et al., 1980), the molecular weight of 40,000 for the protein

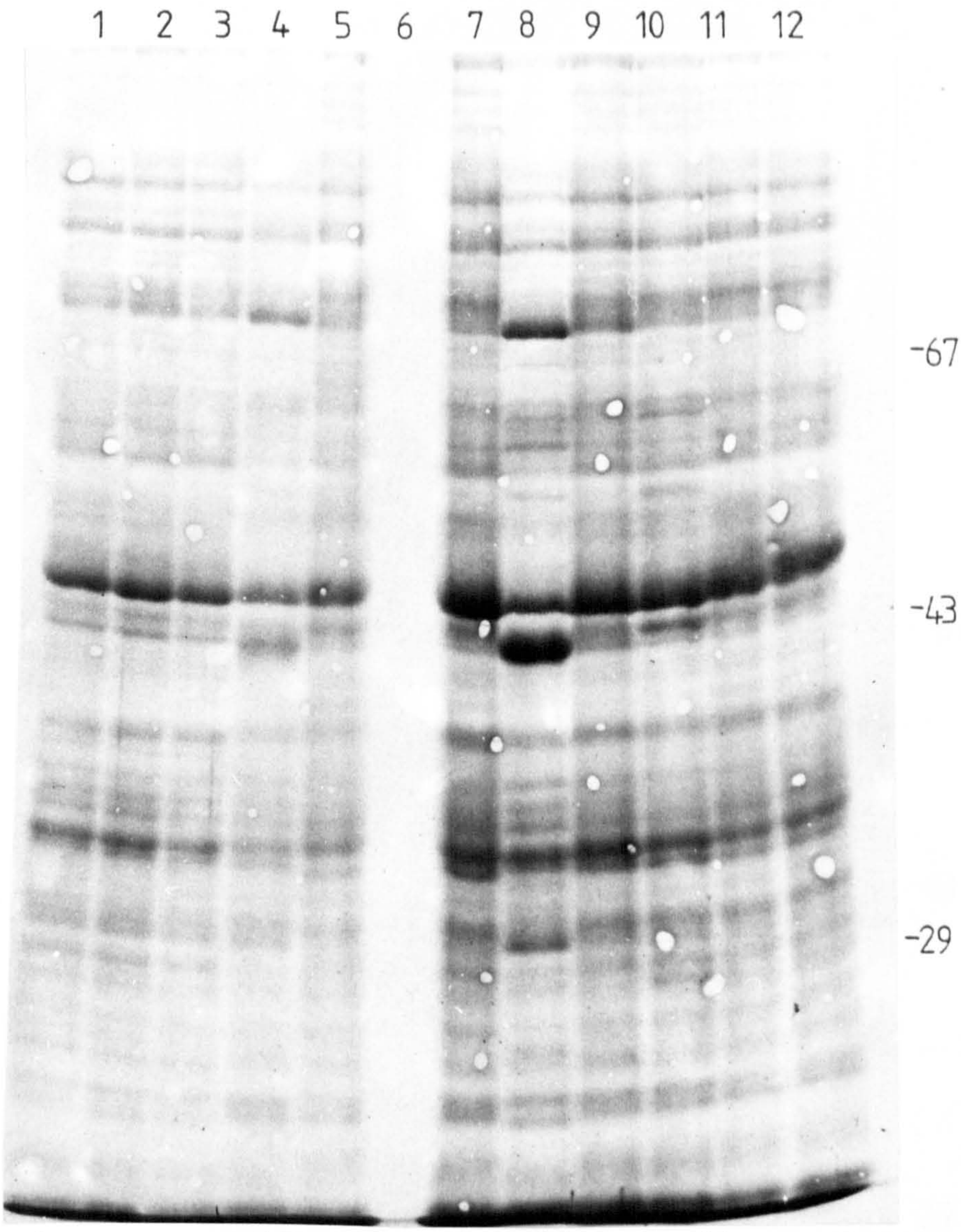


Figure 7.2 Patterns of Protein Synthesis in E.coli Transformed by pWT211 and pSC213.

Total E.coli protein was subjected to electrophoresis on a 10% polyacrylamide gel in NaDodSO4. The protein was transferred to nitrocellulose and stained with amido black. Lanes 1-5: E.coli protein from cultures at A600=0.5. Lanes 7-12 : E.coli protein from cultures at A600=1.0. Lanes 1 and 10 protein from pWT211 transformed E.coli, repressed; Lanes 2 and 12 derepressed; and Lane 11 induced; Lanes 3 and 7 protein from pSC213 transformed E.coli, repressed; Lanes 4 and 8 induced; and Lanes 5 and 9 derepressed. Lane 6 (^{14}C) labelled protein markers. Molecular weights indicated in daltons $X10^{-3}$.

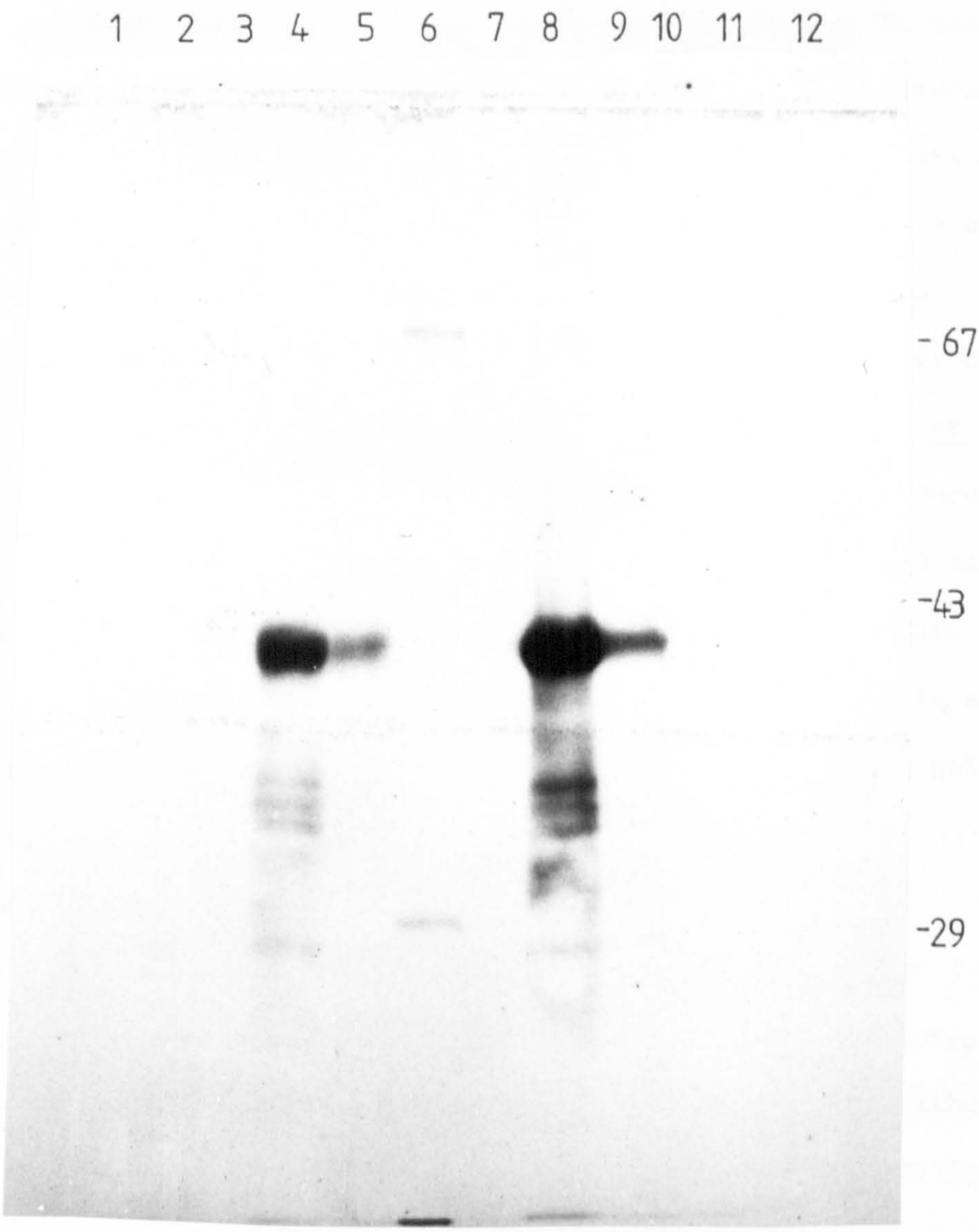


Figure 7.3 Patterns of Human ε Chain Synthesis in E.coli Transformed by pWT211 and pSC213.

Total E.coli protein was subjected to electrophoresis as in Figure 7.2, and proteins containing Human Ig ε antigenic determinants identified using [^{125}I] labelled anti-human Ig ε, Ig G. The molecular weights of the standard proteins are indicated in daltons X10^{-3}.

band and major antigenic activity was in reasonable agreement.

The demonstration of the production of Ig ε CH_3 and CH_4 domain antigenic determinants expected for the expression of the "Fc gene" fragment, under the control of the tryptophan operon, demonstrated the success of cloning this fragment. In Figure 7-3, in addition to the major antigenic determinate at 40,000 daltons, there are also lower molecular weight polypeptides. These could arise due to proteolysis, incomplete translation or truncated translation products due to incorrect initiation of polypeptide synthesis (Gheysen et al., 1982). The track in Figure 7-3 containing the products of induced expression of Trp E/Ig ε taken at absorbance (A) 600 nm of 1.0 also shows higher molecular weight material on longer exposures. This is probably due to the aggregation of small amounts of the protein, as can be found for other polypeptides. The use of a differing gel system may remove this problem, but at this level it is not a significant problem.

Initial results with material prepared by cleared lysates (as plasmid preparation method (25)) indicated that the expressed Trp E/Ig ε was largely insoluble. This was not expected because, although Ig G γ heavy chains are insoluble (Stevenson and Dorrington, 1970), Ig E ε heavy chains are known to be soluble under physiological conditions (Dorrington and Bennich, 1978). The carbohydrate (16.4%) on human Ig E ε heavy chains with a level of 18.5% for the Fc region (Ishizaka and Ishizaka, 1975) compared to Ig G Fc of 6-9% (Nisonoff et al., 1975) may be responsible for the solubility of Ig ε. The bacterially expressed product, devoid of carbohydrate

may as a result have the characteristic solubility properties of Ig G γ heavy chains. The insolubility of the expressed Trp E/Ig ε was investigated in more detail by disrupting the bacteria by sonication under various conditions. The bacterial cultures were grown to an A_{600} of 2 and 10 mL harvested. The pelletsof bacteria were sonicated in 2 mL of 20 mM Tris-HCl pH 8.0, 5 mM EDTA 1 mM PMSF and 5 mM NEM (T.E.P.N.), T.E.P.N. and Triton X 100 1% (v/v), and T.E.P.N. and isopropyl alcohol 10% (v/v). The sonicates were cleared at 16,000 g for 20 minutes at $4^{o}C$ (18,000 rpm Sorvall 5534) and the supernatants and pellets analysed on a 12% polyacrylamide/$NaDodSO_4$ gel. The result demonstrated the Trp E/Ig ε was insoluble under these conditions with a good purification from the majority of the E. coli proteins being obtained see Figure 7-4. As the sonication with the detergent Triton X 100 and the isopropyl alcohol did not solubize the material significantly, this indicates that a membrane interaction was not the cause of the insolubility. The pellet material from 50 µl of the sonication with 10% (v/v) isopropyl alcohol was subjected to solubization with 20 µl of 2% (w/v) $NaDodSO_4$, or 6 M urea, or 2% (w/v) $NaDodSO_4$ in 6 M urea, at room temperature with mixing for about 5 minutes. These extracts were spun as above for 30 minutes in order to remove any insoluble material. The pellets and supernatants from these extractions were analysed by gel electrophoresis (Figure 7-5) demonstrating the solubization of the pelleted material by all the above solutions. The electrophoretic pattern of the pellet produced by 2% (w/v) $NaDodSO_4$ alone was unusual, inconsistant with apparent solubization of all the

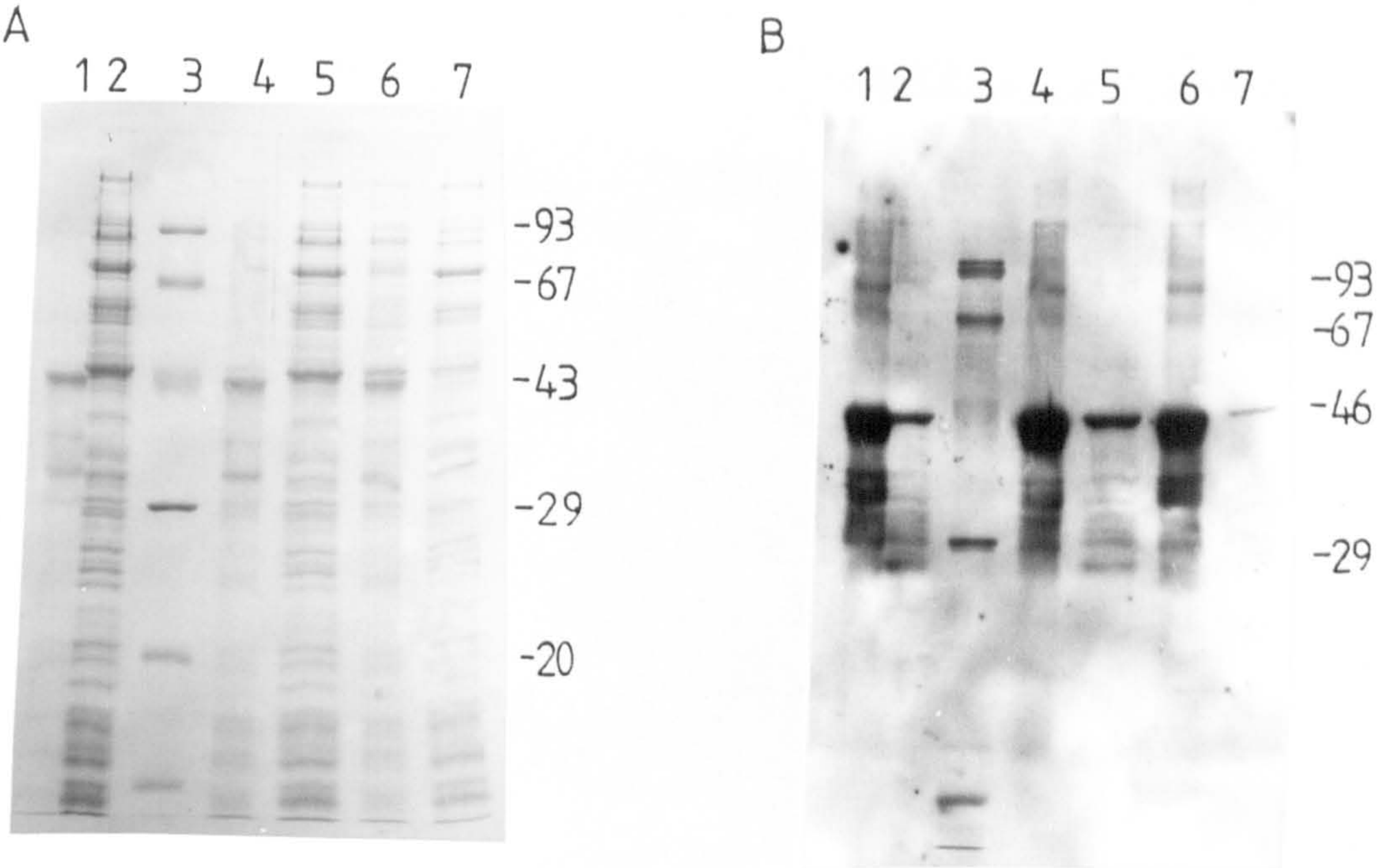


Figure 7.4 Patterns of Proteins Synthesized in E.coli WT217 Transformed with pSC213 and Induced with 3-β-indole acrylic acid in Pellets and Supernatants from Sonications.

A) E.coli proteins were subjected to electrophoresis on a 12% polyacrylamide gel in NaDodSO4 the proteins transferred to nitrocellulose and stained with amido black. Lanes 1, 4, 6 pellets; and lanes 2, 5, 7 supernatants from sonications. Lanes 1 and 2: sonication in 10% isopropyl alcohol TEPN. Lanes 4 and 5: sonication in 1% Triton X100 TEPN. Lanes 6 and 7: sonication in TEPN. Lane 3 protein markers. TEPN is 20mM Tris-HCl pH8, 5mM EDTA, 1mM PMSF and 5mM NEM.

B) Proteins as in A) identified as human Ig ε antigen containing by [^{125}I] labelled anti human Ig ε, Ig G. Molecular weights indicated in daltons X10^{-3}.

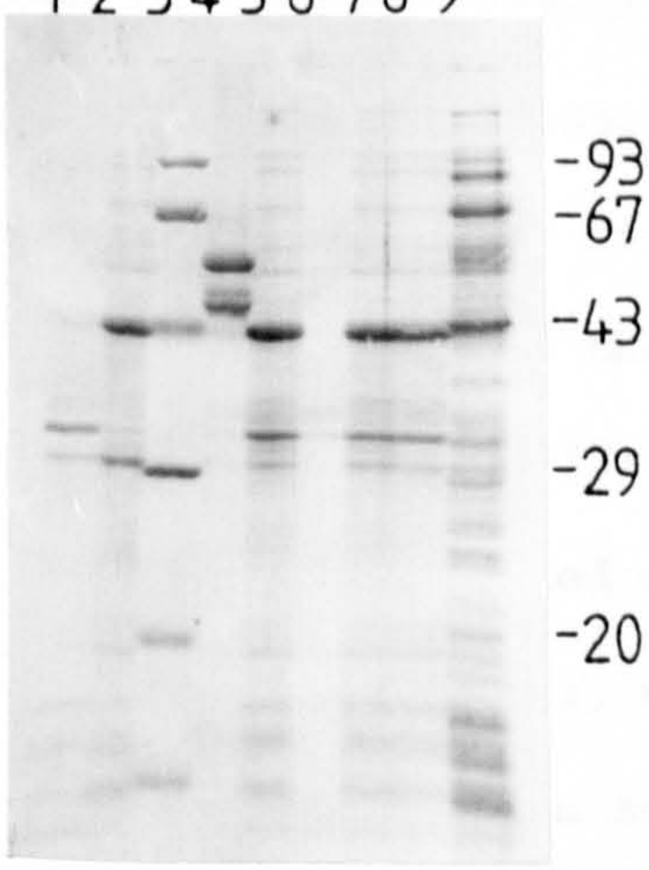


Figure 7.5 Solubilization of Proteins insoluble from Sonication of E.coli in 10% isopropyl alcohol TEPN as in Figure 7.4. E. coli proteins were subjected to electrophoresis on a 12% polyacrylamide gel in NaDodSO4 the proteins transferred to nitrocellulose and stained with amido black. Lanes 1 and 2, pellet and supernatant respectively from solubilization in 6M urea. Lanes 4 and 5, pellet and supernatant respectively from solubilization in 2% NaDodSO4. Lanes 6 and 7, pellet and supernatant respectively from solubilization in 6M urea, 2% Na DodSO4. Lanes 8 and 9, pellet and supernatant respectively from sonication of E.coli in 10% isopropyl alcohol TEPN. Lane 3, protein markers. Molecular weights indicated in daltons $X10^{-3}$.

protein from the pellet to judge by the gel pattern of the supernatant. This must have been due to contamination, as all samples were denatured in the same way before loading the control pellet does not exhibit the anomalous protein bands. In further investigations it was assumed that the insolubility of the Trp E/Ig ε expression product was a characteristic of the Ig ε as found for other Ig heavy chains (Nisonoff et al., 1975). The insolubility of the Trp E/Ig ε product was then investigated with the methods used for maintaining solubility of Ig γ heavy chains, e.g. 1 M propionic acid. This acts by maintaining a low pH (2.8) and solvating with its detergent like activity (Nisonoff et al., 1975). Pellets obtained from the sonicated bacteria using T.E.P.N. and 10% (v/v) isopropyl alcohol were extracted with 6 M or 3 M urea, or 1 M or 0.5 M propionic acid. The results (Figure 7-6) with pellets and supernatants from these extractions shows that the 3 M urea and 0.5 M propionic acid do not solubize the Trp E/Ig ε product, but the 3 M urea appeared to extract some material of higher molecular weight than the Trp E/Ig ε band. The 1 M propionic acid and 6 M urea appeared to extract the Trp E/Ig ε Material, the 6 M urea extracting the majority of this material together with some purification as small molecular weight material was removed. The 1 M proprionic acid appeared to extract the Trp E/Ig ε material preferentially (Figure 7-6). Also shown with this data is Trp E/Ig ε solubized and electrophoresed without reduction with 2-mercaptoethanol indicating that most of the Trp E/Ig ε was not dimerised as expected for an Fc structure or polymerized through disulphide linkages resulting in insolubility.

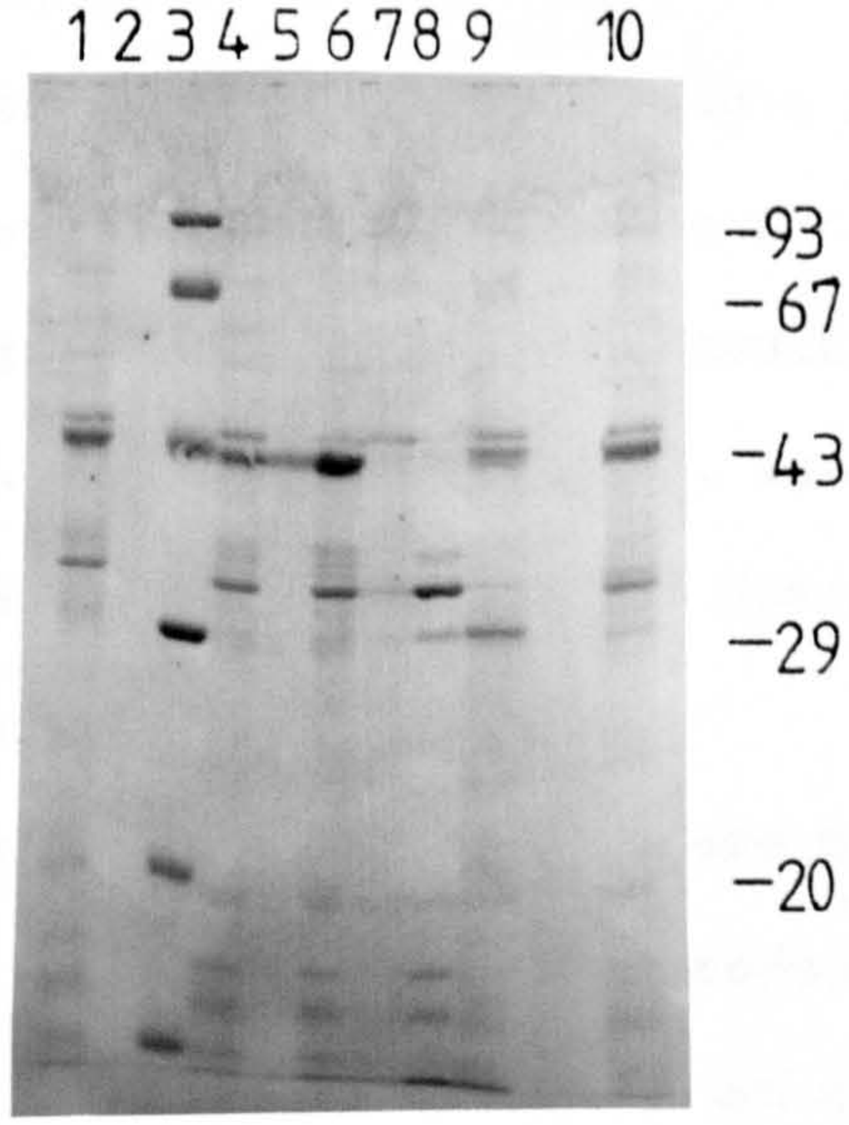


Figure 7.6 Solubilization of Proteins insoluble from Sonication of E.coli in 10% isopropyl alcohol TEPN as in Figure 7.4 E.coli proteins were subjected to electrophoresis on a 12% polyacrylamide gel in NaDodSO4 the proteins transferred to nitrocellulose and stained with amido black. Lane 10: pellet material from sonication solubilized in loading buffer without 2-mercaptoethanol. Lanes 1 and 2: pellet and supernatant respectively from solubilization with 0.5M propionic acid. Lanes 4 and 5: pellet and supernatant respectively from solubilization with 1M propionic acid. Lanes 6 and 7: pellet and supernatant respectively from solubilization using 3M urea. Lanes 8 and 9: pellet and supernatant respectively from solubilization using 6M urea. Lane 3: protein marker molecular weight indicated in daltons $X10^{-3}$.

The observation of Williams et al., (1982) of accumulation of Trp fusion expression products, together with our initial observation of the increase in the level of the Trp E/Ig ε protein, led to an investigation of the production of Trp E/Ig ε after induction. The results (see Figure 7-14 for typical result) showed that the Trp E/Ig ε product accumulated, suggesting, as also demonstrated by Williams et al., (1982), that there may be discrete aggregations of protein (inclusions) within the bacteria.

In order to study the expression in more detail, development of an assay for the insoluble Trp E/Ig ε product, based on the available antibodies was necessary. The use of materials capable of solubising proteins are usually denaturants of proteins and would also disrupt antigen-antibody interactions which form the basis of the assay. Thus in development of the assay we needed to find a method for solubisation of total bacterial protein which also lysed the bacteria, avoided involved sample handling, and allowed sufficient dilution of the solubization medium to allow antigen-antibody binding. The amount and nature of the medium required to solubilize the Trp E/Ig ε from the bacteria will affect the degree of dilution needed to allow antibody-antigen interaction. The dilution required for this interaction may dilute the antigen to below the sensitivity of the assay urea and NP40 are used to produce denaturing conditions in isoelectric focussing (O'Farrell et al., 1977), accordingly these agents, low pH and Na Dod SO_4 were tested.

In order to arrive at an initial lysis mix studies were made of the effect of these agents on the antigen/antibody binding, using DAKO anti human Ig E ε chain specific rabbit Ig G. These antibodies were chosen in preference to TAGO affinity pure goat anti human ε Ig G, chain, as it was a possibility that a whole Ig G preparation might stand up better to denaturing conditions. This was used in a 'sandwich' with (^{125}I) labelled antibodies essentially as described for the ELISA (13). The samples were diluted in differing buffers, as described later, and flexible Vinyl (Falcon plastics) microtiter dishes were used to facilitate the cutting out of wells for counting. The material used in evaluation of the effects of sample dilution buffers were culture supernatants from the cell line 266BL, providing human Ig E. The results (Table 7-1) indicated that up to 4M urea, 0.3% NP40, and 5 mM CH_3COONH_4 pH 4.5, could be used without dramatically altering the binding capacity of the antibodies. This result led to the use of 9 M urea, 0.5% NP40, 10 mM EDTA, 5 mM PMSF, 10 mM NEM and 5 mM CH_3COONH_4 pH 4.5 to lyse the bacteria, followed by heating at 95°C for 5 minutes. (This mix was used at 95 µL for bacterial pellet derived from the equivalent of 1 mL of culture at 1 absorbance 600 nm (A_{600} mL).

The analysis of this bacterial lysate on gels, (Figure 7-7) before centrifugation and the supernatants indicated that some of the Trp E/Ig ε had not been solubilized. The bacterial lysate with its significant amount of soluble Trp E/Ig ε was tested in the assay with (I^{125}) Ig G but even at lower dilutions of sample

Sample Buffer	CPM		% Binding of
	Test	Background	PBS Value
PBS	375	81	100
5mM NH_4Ac pH 4.5			
+ 0.06M urea	322	70	86
+ 0.15M urea	306	80	77
+ 0.6 M urea	329	80	85
+ 1.5 M urea	250	74	60
+0.1% NP40, 1.5M urea	313	79	80
+0.2% NP40, 2M urea	281	88	66
+0.2% NP40, 3M urea	261	84	60
+0.3% NP40, 4M urea	261	83	61
+0.02% $NaDodSO_4$, 0.06M urea	78	-	-
+0.05% $NaDodSO_4$, 0.15M urea	99	-	-
+0.2% $NaDodSO_4$, 0.6M urea	101	112	-
+0.5% $NaDodSO_4$, 1.5M urea	97	90	-

Test sample 4 µL of culture medium RPMI 1640, 10% FCS from a culture of 266BL, background 4 µL of culture medium before 266BL culture.

Table 7-1 The effects of various concentrations of urea, NP40 and $NaDodSO_4$ in 5 mM NH_4Ac pH 4.5 on antibody: Ig E binding. The level of Ig E binding was developed using (^{125}I) labelled antibodies in PBS (2×10^4 CPM/Well/100 µl).

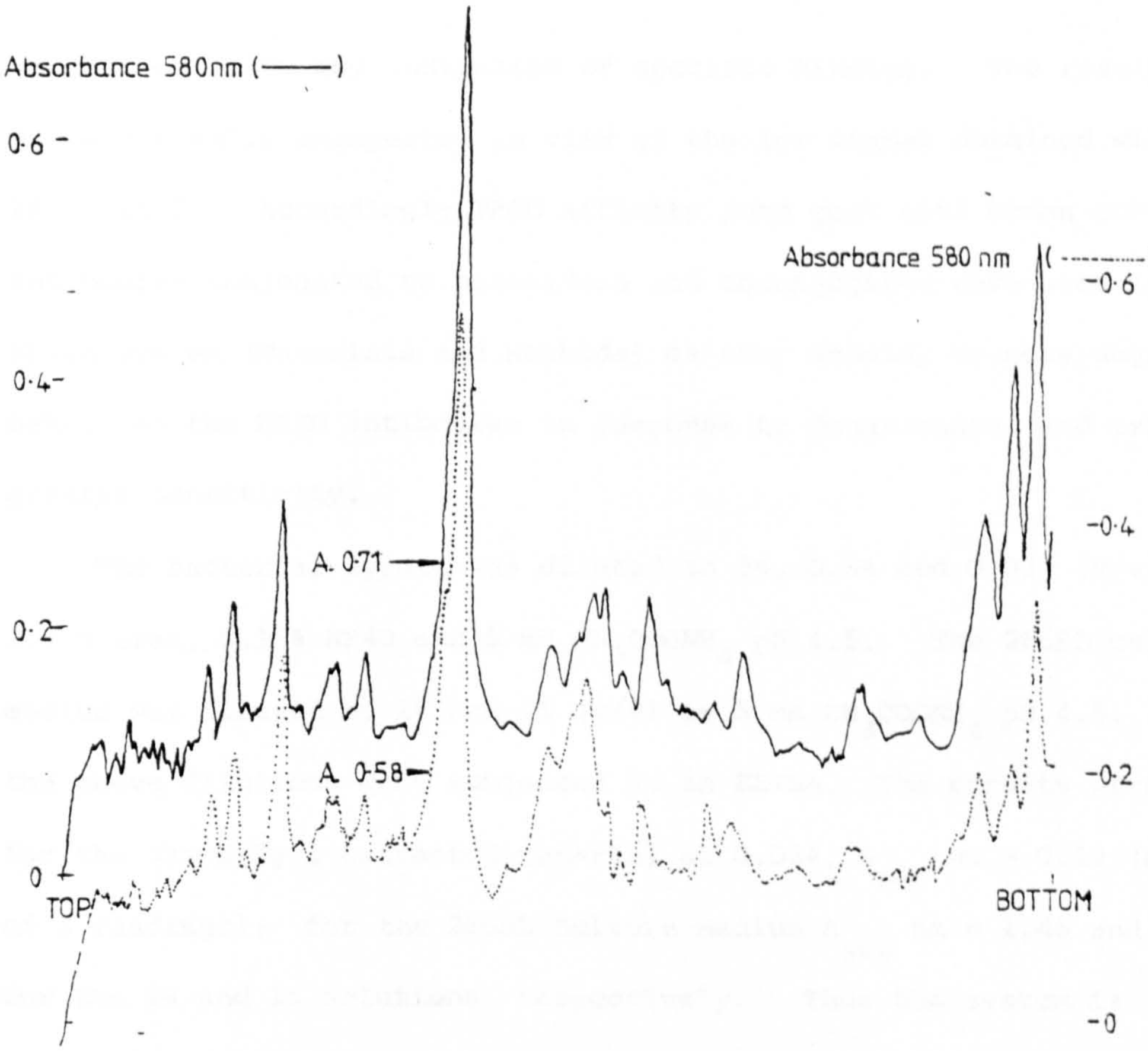


Figure 7.7 Proteins from E. coli WT217 transformed with pSC213 after induction, subjected to solubilization.

The proteins from E. coli were solubilized using 9M urea, 0.5% NP40, 10mM EDTA, 5mM PMSF, 10mM NEM and 5mM CH_3COONH_4 pH4.5 and subjected to electrophoresis on a 12% polyacrylamide gel in NaDodSO4. The gel was stained with Coomassie Blue G250 and scanned at 580nm. The top trace (solid line) shows total protein and the lower trace (dotted line) shows soluble protein from a solubilization on an equivalent amount of total protein. The peak absorption of the Trp E/Ig ε peak is given for estimation of the level of solubilization.

it did not show any indication of specific binding. The result was not totally unexpected in view of the low signal obtained with 266BL Ig E. Accordingly TAGO affinity pure goat anti human εchain antibodies conjugated to peroxidase and unconjugated were used in an ELISA system (Materials and Methods) as they should, to some degree, behave as the DAKO antibodies in response to denaturants, and exhibit greater sensitivity.

The bacterial lysate was diluted to 3%, 0.3% and 0.03% (v/v) in 2.7 M urea, 0.15% NP40 and 5 mM CH_3COONH_4 pH 4.5. The 266BL culture medium was diluted to 1% and 2% (v/v) in 5 mM CH_3COONH_4 pH 4.5. All the above dilutions were subjected to an ELISA. The results were: for the Trp E/Ig ε extracted material at 0.03%, A_{540} nm = 0.19 (average of 3 readings); for the 266BL culture medium A_{540} nm = 1.46 and 1.0 for the 2% and 1% solutions, respectively. Thus the system is sufficiently sensitive to allow detection of levels at least over a 1,000 fold range in the present solubization buffer.

The range of sensitivity available and the incomplete solubization of the Trp E/Ig ε led to the re-examination of the effects of Na Dod SO_4. To examine Na Dod SO_4 three separate doubling dilutions were made from an initial 0.3% dilution of the bacterial lysate (as described above), but with one now containing 0.01% and another 0.1% Na Dod SO_4. The presence of 0.1% Na Dod SO_4 completely eliminated any reaction but at 0.01% Na Dod SO_4, with an 8 fold dilution of sample, the signal was 44% of the sample in the absence of Na Dod SO_4. The high dilutions of sample in 0.01% Na Dod SO_4 brought about

greater reductions in A_{540} relative to the samples in its absence, possibly due to the lower protein to $NaDodSO_4$ ratio.

The ability of the assay to work in the presence of some $NaDodSO_4$, which is known to improve the solubization of Trp E/Ig ε, led to its inclusion in a revised lysis buffer, viz. 9 M urea, 0.2% (w/v) $NaDodSO_4$, 0.5% (v/v) NP40, 10 mM EDTA, 2 mM PMSF, 5 mM NEM and 5 mM CH_3COONH_4 pH 4.5. This buffer was used at the rate of 133 µL per A_{600} mL, followed by heating at 100°C for 5 minutes. The samples were diluted in 2.7 M urea, 0.01% (w/v) $NaDodSO_4$, 0.15% (v/v) NP40 and 5 mM CH_3COONH_4 pH 4.5 for assay.

This new lysis mix was then applied to an induction experiment in which cultures of E. coli WT217 containing plasmids pSC213 and pWT 211 were sampled at various times. The material obtained by solubizing the samples was run on a 12% polyacrylamide gel (Figure 7-8) which revealed the soluble and insoluble polypeptides. Apart from two lower molecular weight polypeptides which do not appear to be sufficiently solubized the bacterial protein was adequately solubized for assay.

The samples from the above induction experiment were assayed as described in the absence of the described bacterial extract, with bovine serum albumin (BSA) or casein in the antigen dilution buffer. The plots of these samples from the ELISA (Figure 7-9 A) show that the control extracts gave no background of significance, and the test extracts gave increasing levels of Ig ε antigen with time from induction.

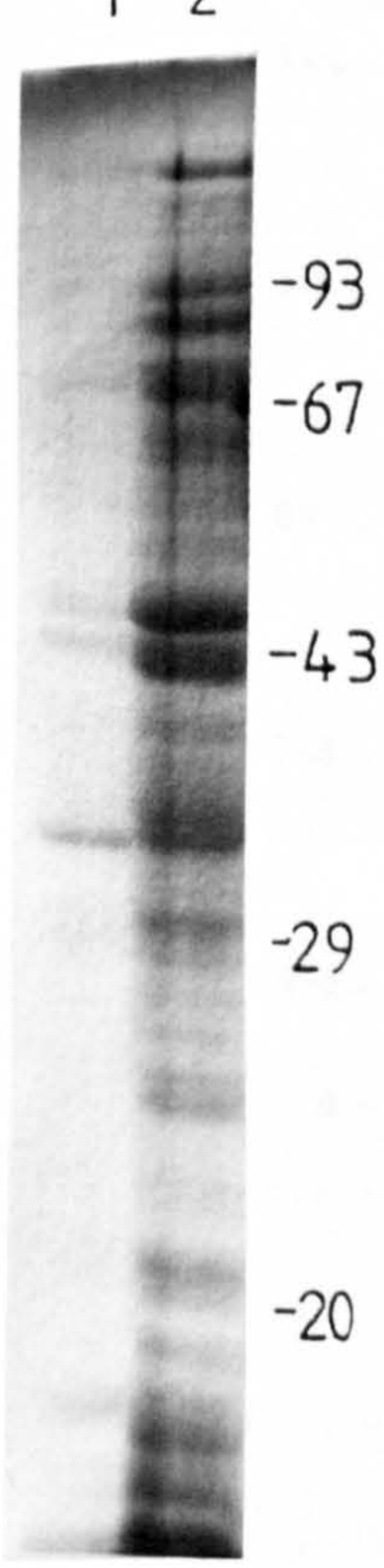


Figure 7.8 Proteins from E.coli WT217 transformed with pSC213 after induction subjected to solubilization.

Lane 1) pellet of protein after solubilization

Lane 2) soluble proteins after using 9M urea, 0.2% NaDodSO4, 0.5% NP40, 10mM EDTA, 2mM PMSF, 5mM NEM and 5mM CH_3COONH_4 pH4.5. Molecular weights indicated in daltons $X10^{-3}$.

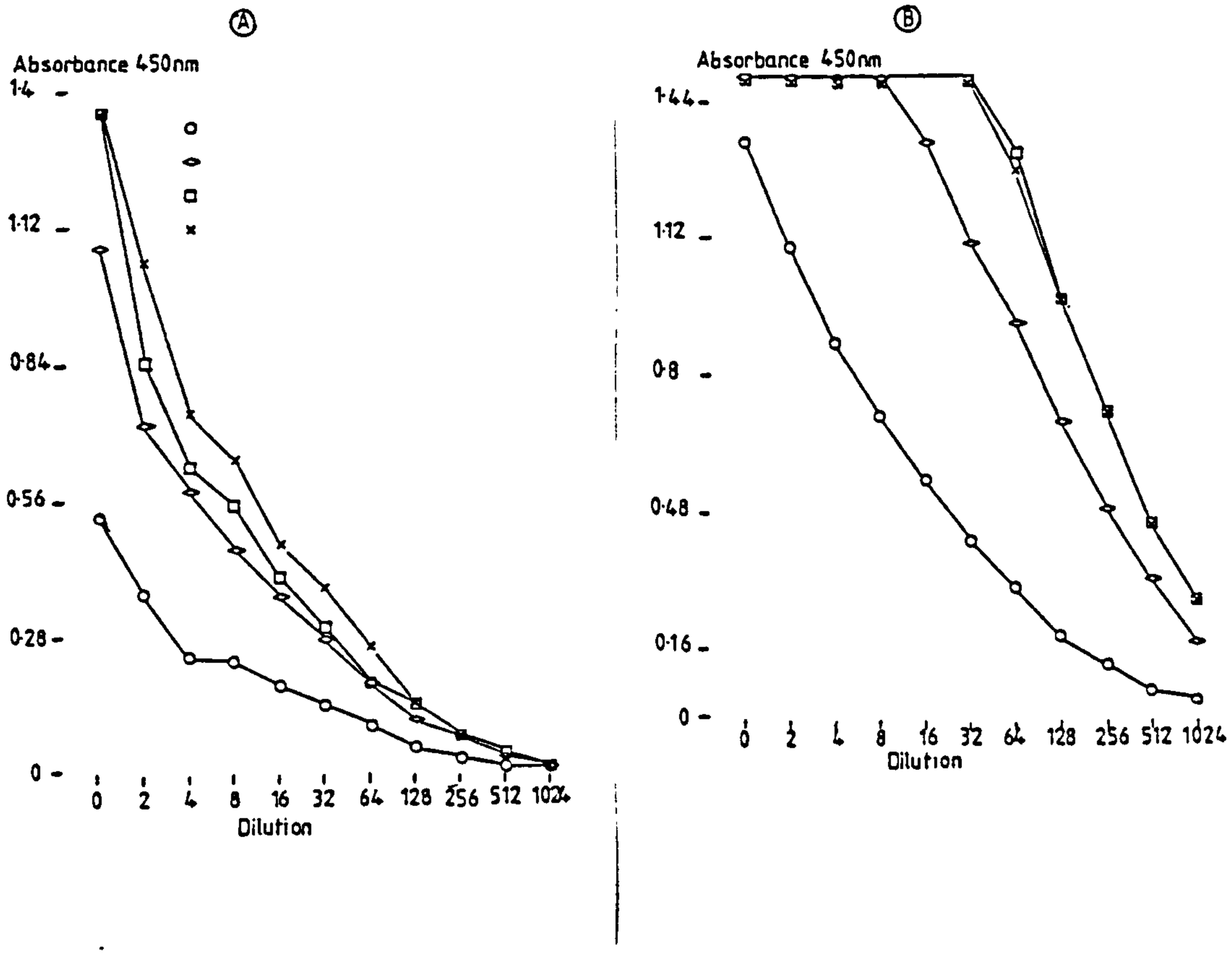


Figure 7.9 Samples from E.coli WT217 transformed with pSC213 during the course of a culture and analysed in the ELISA for Ig ε antigenic determinants.

A) Samples at 0.2 O, 0.5 ◇, 0.8 □, and 1.0 X A600 equal amounts diluted across the assay plate in sample buffer (see text).

B) Samples as in A) but diluted in sample buffer with 5% BSA.

Possibly due to a reduction in the Na Dod SO_4 : protein ratio, adding 5% BSA to the sample dilution buffer caused a marked increase in sensitivity (Figure 7-9A, 7-9B). This addition was made to all subsequent dilution buffers.

The ELISA was found to give differing curve shapes for the doubling dilutions of different samples. This appeared to be dependent on the % of total protein present as Trp E/Ig ε. In an equivalent assay for bacterial expressed β interferon (S. Buckham, personal communication) it was found that a significant amount of binding to the assay plate independent of the coated antibody was taking place. Similar binding was found in the ε assay and was more significant as the level of Trp E/Ig ε rose during induction. This effect was demonstrated by the plots from the ELISA results, with samples being assayed for binding to normal goat Ig G coated micro-titer wells in addition to the usual anti Ig ε goat Ig G coated wells (Figure 7-10A). This demonstrated binding of Trp E/Ig ε in the absence of specific antibodies to Ig ε. The possibility existed that the binding to the plates was mediated via the casein used for blocking protein binding sites in the initial preparation of the plates, as binding does still occur if no Ig G is present. This idea may be supported by the result found for β interferon (S. Buckham personal communication) that inclusion of casein in the sample dilution buffer removed the effect. In our study of the binding the lower percentages of Trp E/Ig ε suffered less from this problem. Therefore casein and a control bacterial extract were included such that dilution of only the expressed product occurred

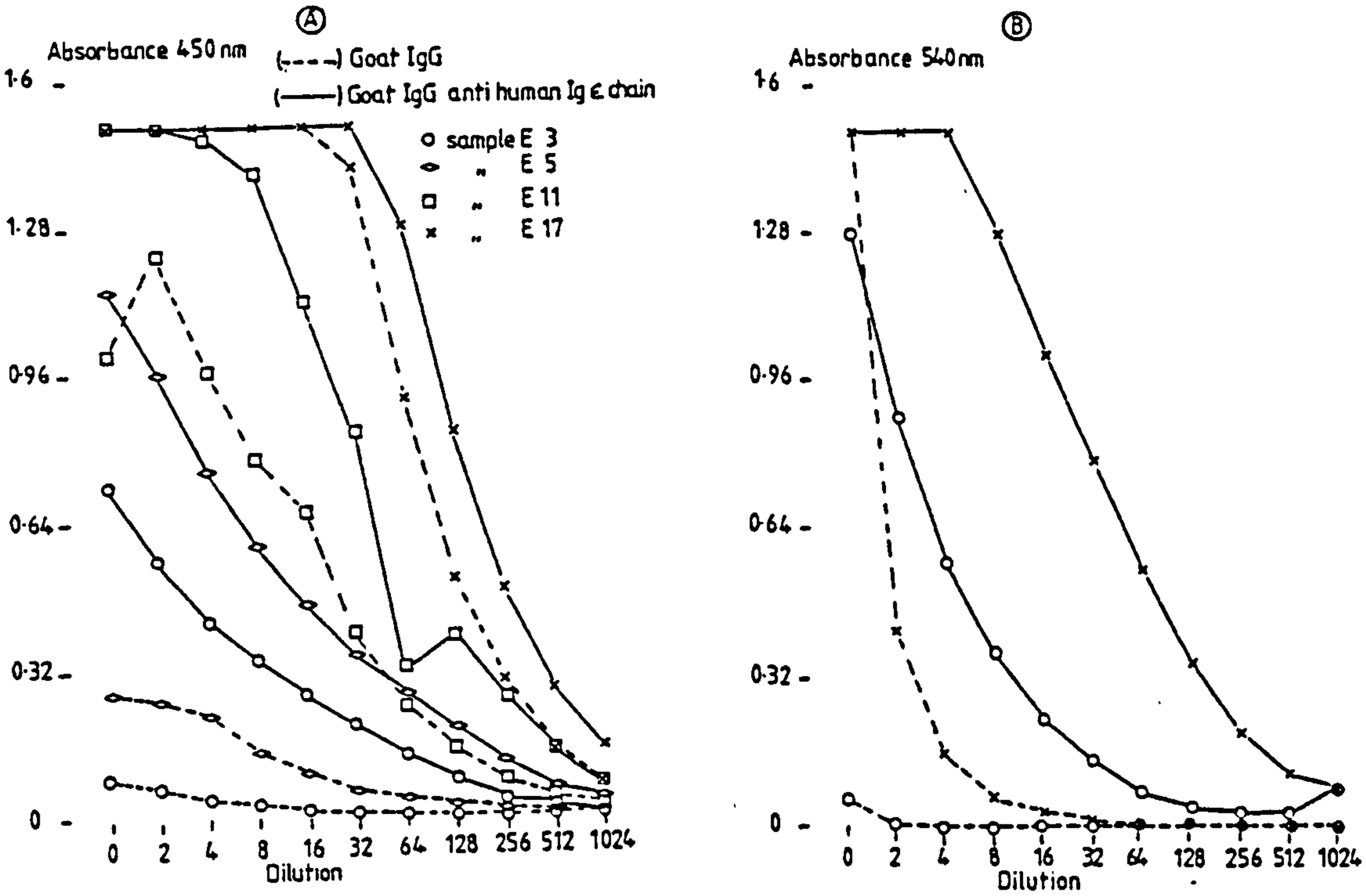


Figure 7.10 Samples from E.coli HB101 transformed with pSC213 during the course of a culture and analysed in the ELISA demonstrating the nonspecific binding of Ig ε antigenic determinants and its inhibition.

A) Samples at culture A600's of 0.3, E3; 0.5, E5; 1.2, E11; 3.0, E17 equal amounts diluted across the assay plate pre-coated with normal goat Ig G or anti human Ig ε chain goat Ig G in sample buffer without casein or bacterial extract.

B) Samples E3 and E17 as in A) but diluted in sample buffer containing in addition 0.5% casein and 5% bacterial extract (see materials and methods).

against a constant level of bacterial extract.

The results with the improved dilution buffer are shown for two selected samples from figure 7-10A in 7-10B, demonstrating the effectiveness in removing the non-specific binding of the Trp E/Ig ε antigen.

The application of this modified assay to expression of plasmid pSC213 in HB101 demonstrated the rapid synthesis of Ig ε antigen after addition of 3 β indole acrylic acid. The level of Ig ε antigen correlated with the production of the protein band at 40,000 daltons previously identified as containing the Ig ε antigen (see figures 7-11 to 7-15).

The expression particularly with HB101 was at such a high level, that investigations into the morphology of the bacteria was carried out by electron microscopy of sections of the bacteria, as done by Williams et al., (1982) and Chapman and Cook (G. D. Searle).

In order to examine possible inclusion bodies in more detail it was hoped to immunologically identify them. This was performed as described (36), results are shown in Figure 7-16, demonstrating significant binding of ferritin mediated via goat anti Ig ε, Ig G to defined areas within the E. coli HB101 containing pSC213, not found on reaction of equivalent sections with non-immune goat Ig G. Sections

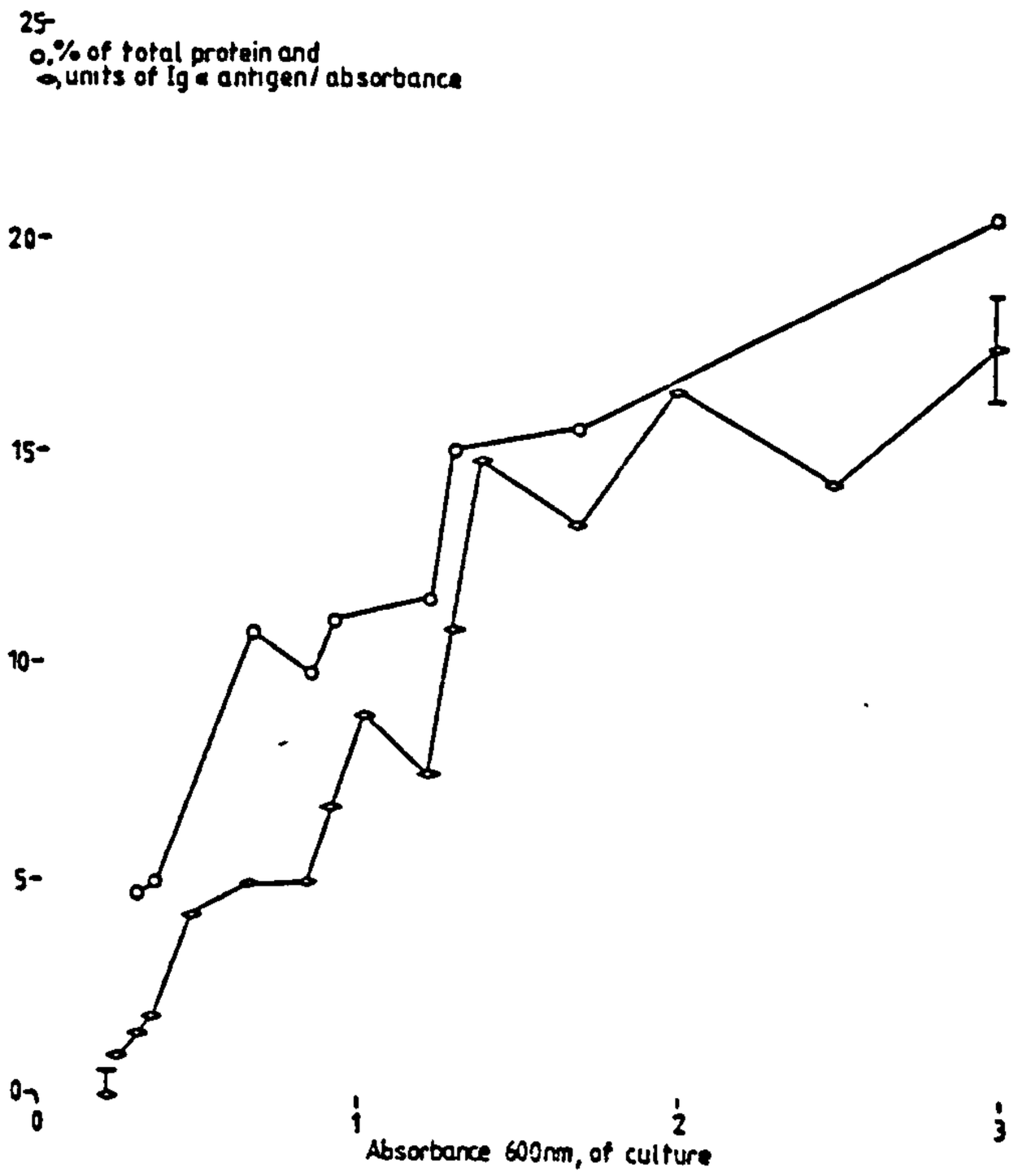


Figure 7.11 Assays of the TrpE/Ig ε chain levels as a function of culture A600.

The relative amounts of the Trp E/Ig ε in E. coli HB101 at varying A600 estimated by scanning Coomassie Blue G250 stained gel shown in Figure 7.14 at 595nm (circles) and by ELISA (Diamonds) are compared. The units for the ELISA assay are arbitrary all assay plates being referenced to the sample obtained at culture A600, 3.0, and set at 17 units. The error bar on the sample at culture A600 of 3.0 is the standard deviation observed for this sample from a number of separate assays (5).

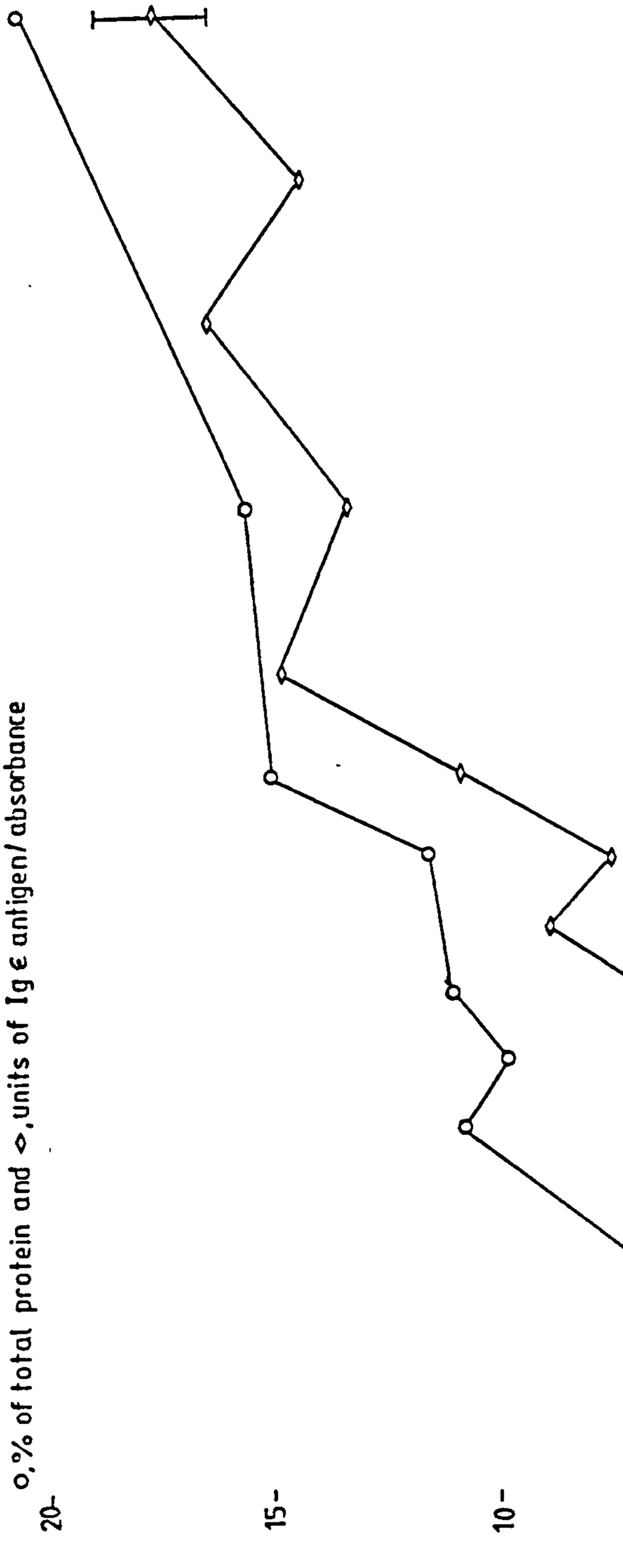


Figure 7.12 Assays of the Trp E/Ig ε chain levels as a function of culture time.

The relative amounts of Trp E/Ig ε in E. coli HB101 at varying times from induction estimated as in Figure 7.11

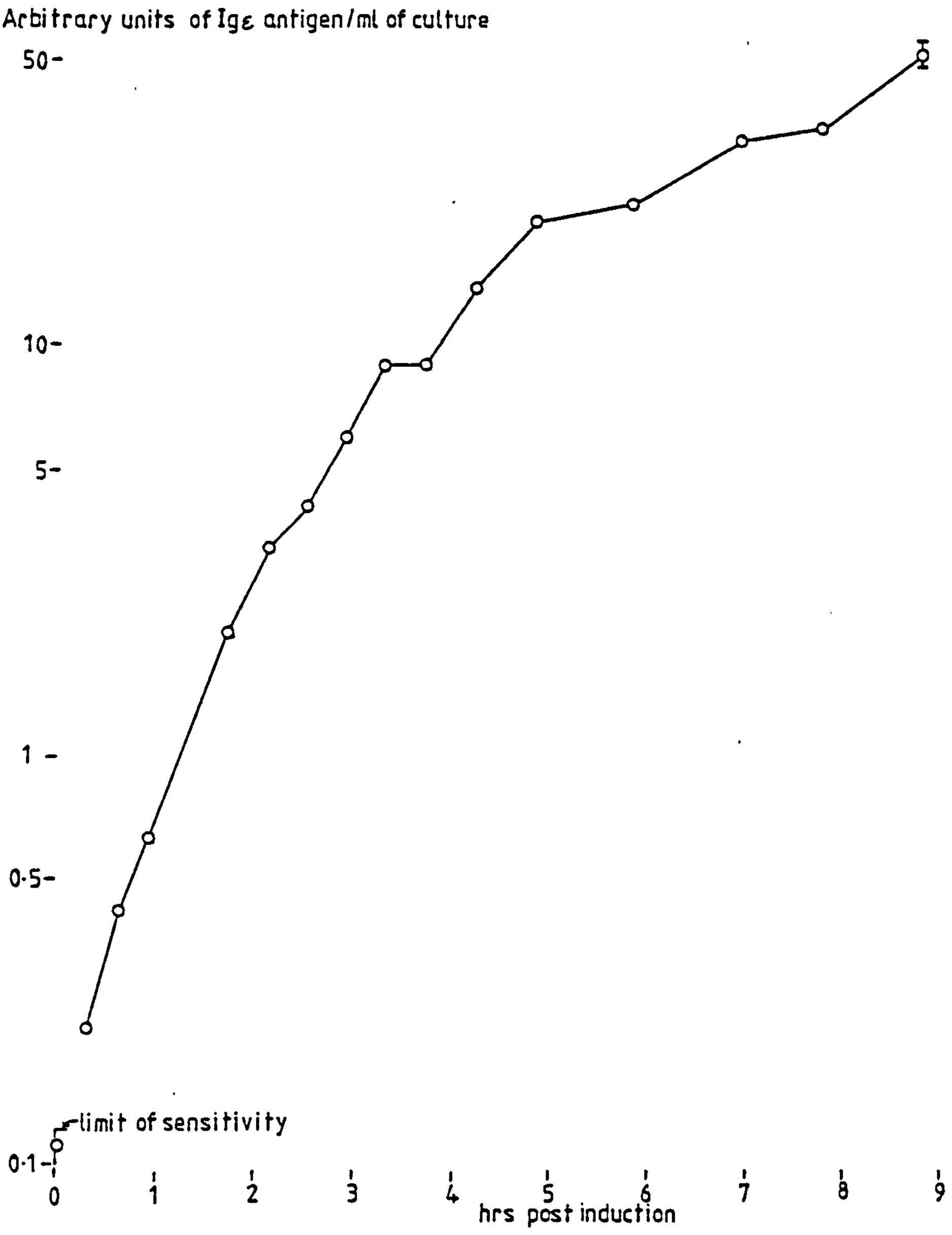


Figure 7.13 Level of the Trp E/Ig ε chain produced as a function of culture time.

The relative amounts of Trp E/Ig ε in culture at varying times from induction estimated by ELISA as in Figure 7.11.

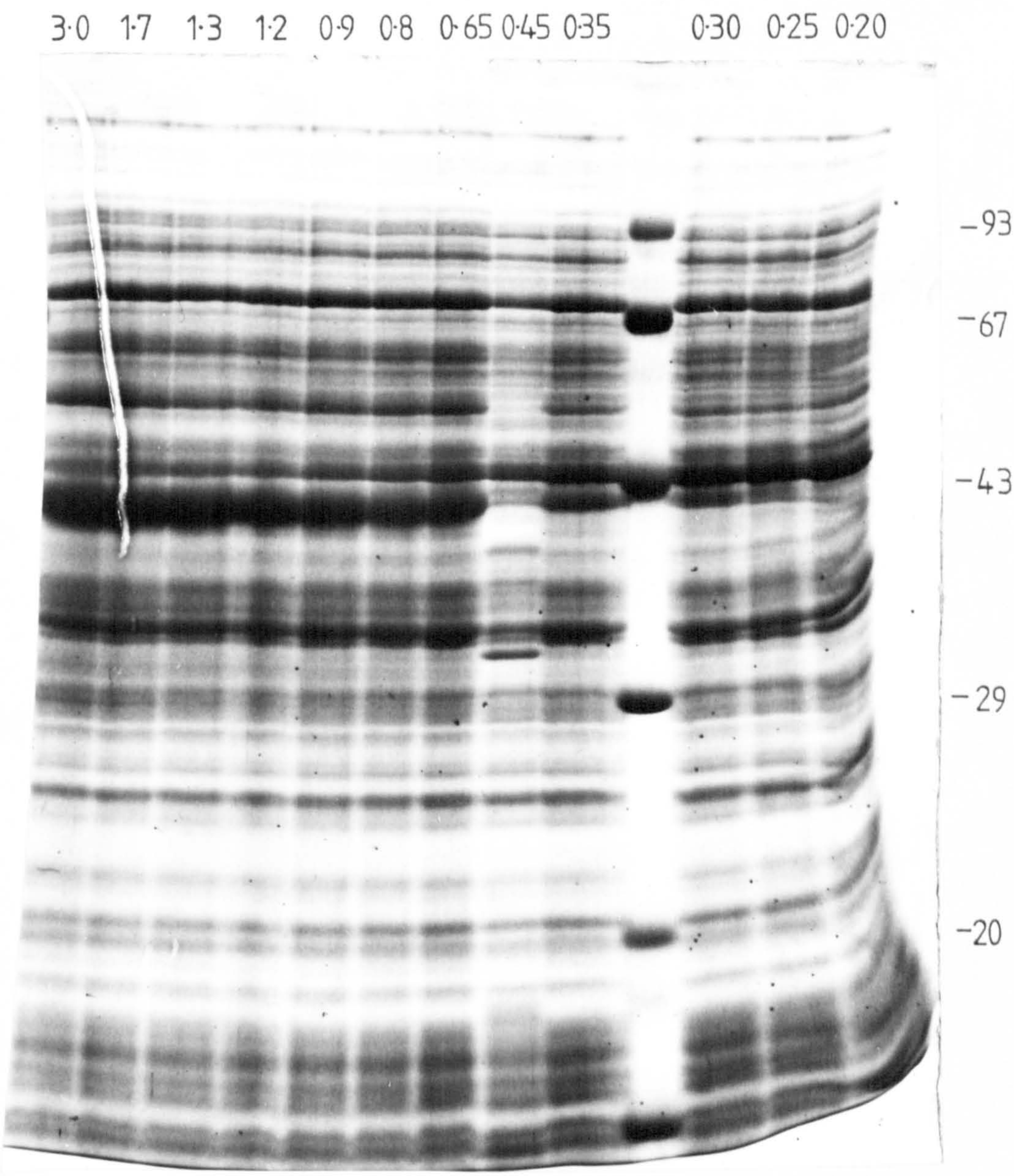


Figure 7.14 Total protein from E.coli HB101 transformed with pSC213 at indicated culture A600.

Protein from E.coli HB101 induced with 3-β-indole acrylic acid at culture A600 0.2 and harvested at the indicated A600 were subjected to electrophoresis on a 12% polyacrylamide gel in NaDodSO4 and subsequently stained with coomassie blue G250. The molecular weights of protein markers indicated in daltons $X10^{-3}$.

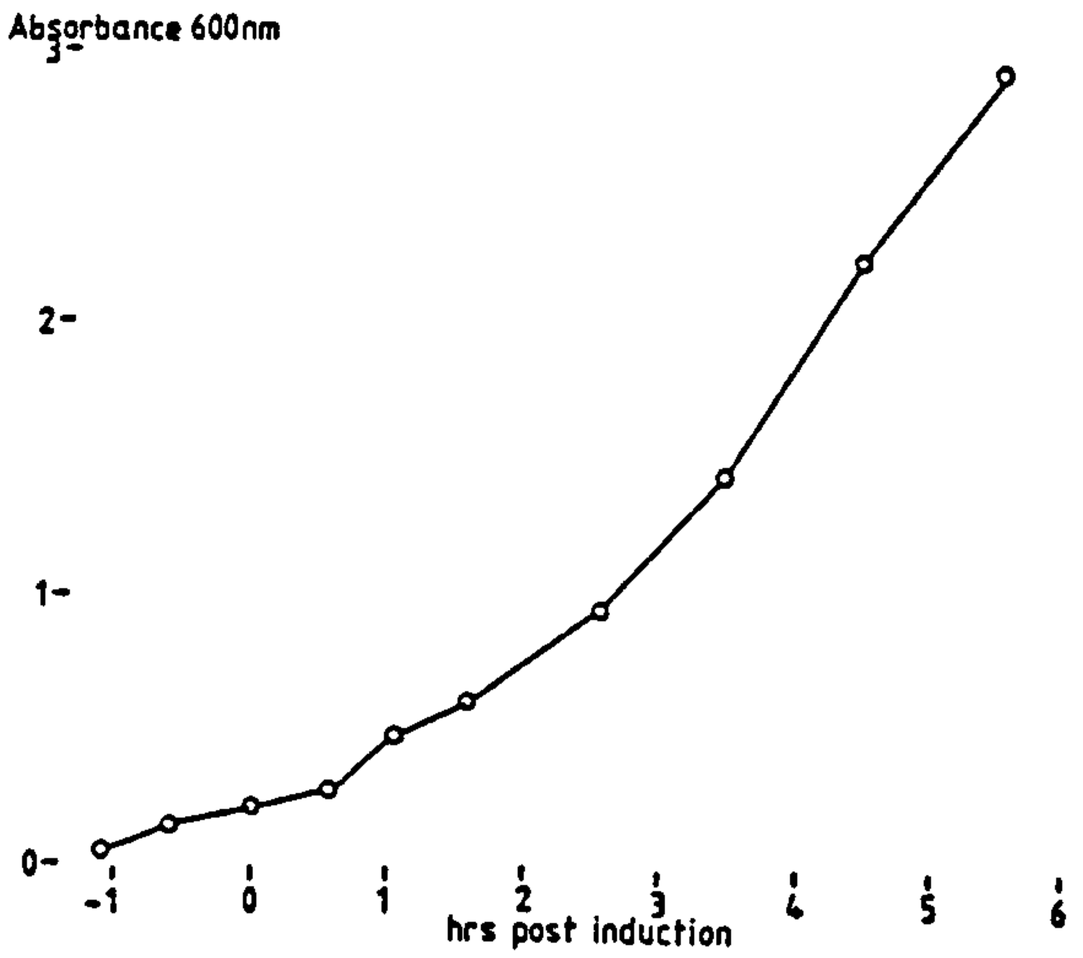


Figure 7.15 Growth rate of cultures of E.coli HB101, transformed with pSC213 (A) and pWT211 (B) after induction.

A

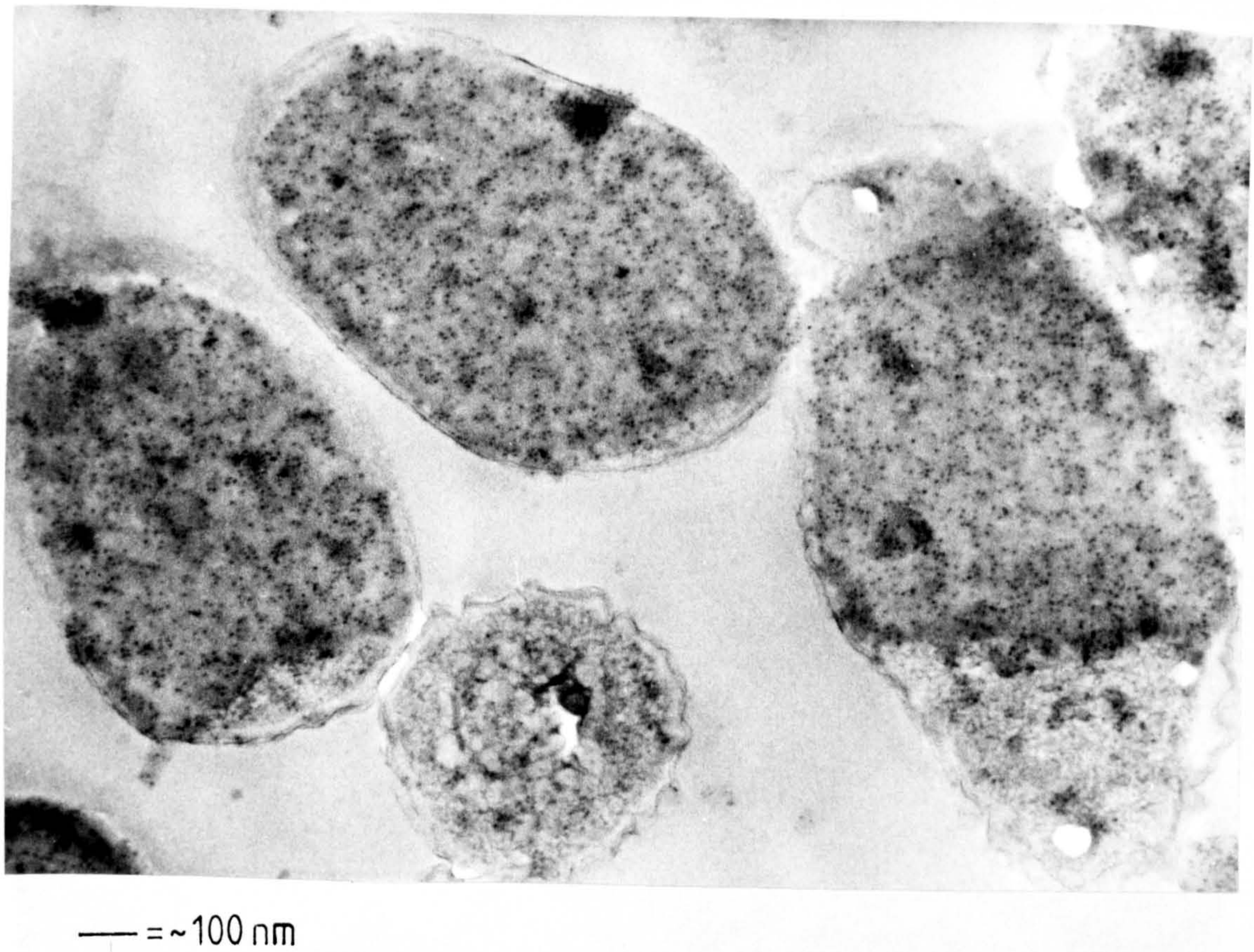
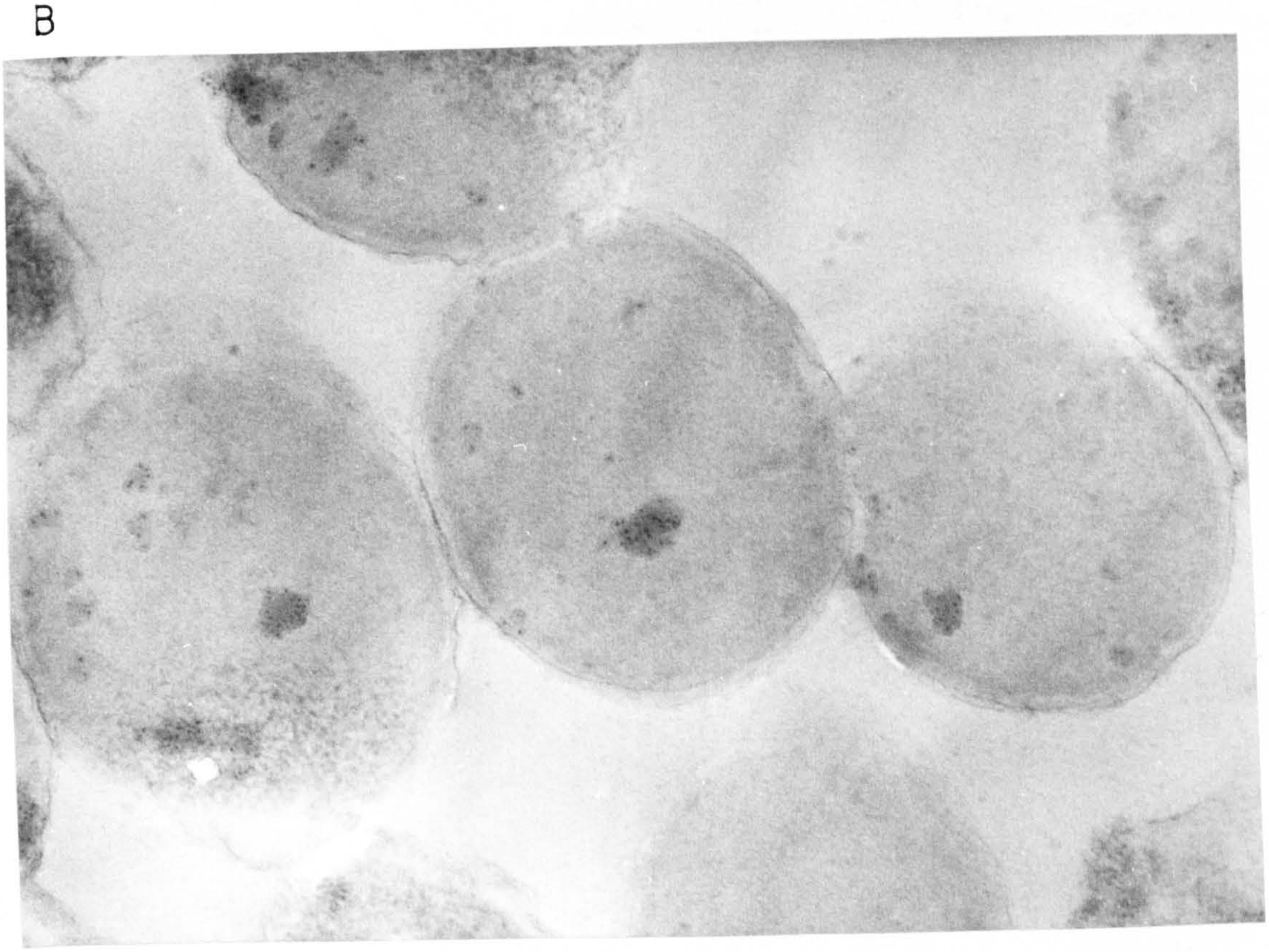


Figure 7.16 Immunoelectron microscopy of E.coli HB101 transformed by pSC213.

A) Bacterial cultures were induced at culture A600 of 0.2 and harvested at a culture A600 of 3.0. Bacterial sections were reacted sequentially with goat anti-human ε chain antibody and ferritin-labelled rabbit anti-goat Ig G.

B) Bacterial sections similar to those described in A) were incubated sequentially with normal (non-immune) goat Ig G and then ferritin-labelled rabbit anti-goat Ig G. PTO.

B



—— = ~100 nm

of E. coli HB101 containing pWT211 did not indicate the presence of inclusion bodies, but these sections were poorly preserved. These sections failed to show any significant binding of ferritin mediated via goat anti Ig ε Ig G.

In summary, the bacterial expressed Trp E/Ig ε product is evidently synthesised at a rate which exceeds its degradation, leading to the accumulation of the product with time in an induced culture. This apparently results in production of aggregates of proteins, taking up a significant amount of the intra-cellular volume. This property is clearly of great value in purification allowing concentration of the expressed product in the bacteria, followed by purification from the majority of E. coli proteins and nucleic acid after sonication by pelleting out from the sonicate by centrifugation.

The solubility of the Trp E/Ig ε in 6 M urea and in 1 M propionic acid shows that the aggregated protein product is not completely insoluble. The relative insolubility of the Trp/Ig ε product may be the factor influencing the production of large amounts of Trp E/Ig ε as a percentage of bacterial protein, it being likely that an insoluble product is less susceptible to proteolysis.

As the product is apparently not in a dimeric form via disulphide bonds, as expected for an Fc fragment, the regions of the polypeptide involved in the inter chain noncovalent interactions may; (Dorrington and Bennich, 1978) be with other regions of the Ig domains involved in non-specific interactions resulting in

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insoluble aggregates. If this were so reconstitution of 'native' structure may initially require the full denaturation of the Trp E/Ig ε, followed by renaturation of dilute protein solutions (0.02 mg/mL, Goto and Hamaguchi (1982)), reducing the potential for interference by interchain interactions, allowing the refolding of the Ig domains within the polypeptide chain. Subsequent concentration, and or raising the pH (e.g. to 8.0), and or oxygenation, and or oxidation by glutathione (Scheele and Jacoby, 1982) may encourage the formation of the disulphide bridges between the Trp E/Ig ε polypeptide chains, resulting in a Fc like dimer. The attempts at renaturation would require at least some purification in order to remove potentially interfering material. Purity in respect of protein may not be sufficient, as at high levels of expression the number of purification methods may be limited resulting in contamination with other impurities.

Tests of samples for carbohydrate, lipid, phosphates, DNA and RNA may be of value.

If problems are encountered with reconstituting an active product from the above Trp E/Ig ε product the generation of other expressed products might be valuable. Within the criteria of an active Fc fragment, the fragments likely to prove useful would be, the Bgl II - Hind III gene fragment and a gene fragment including the interchain half disulphide at the COOH terminal end of the CH_2 domain. The latter fragment would be the smallest fragment capable of forming a dimer (via the disulphide bond sensitive to reduction) likely to contain receptor binding activity (see Introduction).

Further studies into the nature of the Ig E, receptor binding could be made with an active bacterially synthesised product. The expressed product may be modified by site directed mutagenesis (Zoller and Smith, 1982) or deletions to define the structural requirements for activity.

After completion of this work further studies on the bacterial expressed Trp E/Ig ε product have lead to the production of a Trp E/Ig ε dimer (Dr. Helm unpublished results).

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